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Special Issue Reprint

Harnessing of Soil Microbiome for Sustainable Agriculture

Edited by
Debasis Mitra, Marika Pellegrini and Leonard Koolman

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Guest Editors

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About the Editors

Debasis Mitra

Debasis Mitra, recognized among the Top 2% Scientists in the World (2025) and a distinguished fellow of the SASS and IOASD Society in India, serves as Assistant Professor in the Department of Microbiology at Graphic Era (Deemed to be University), Dehradun, Uttarakhand. Dr. Mitra serves as Founder President of PMMITA Society, a registered entity under the Government of West Bengal (West Bengal Act XXVI of 1961), dedicated to advancing microbiological research and innovation globally. With a robust nine years of research experience, he earned his Ph.D. in Microbiology from Raiganj University, India, in conjunction with the ICAR- NRRI, India. His prior experience also includes tenure at ICAR-NRRI, India, under a project funded by DBT, Govt. of India. Dr. Mitra holds an M.Sc. in Biotechnology from Graphic Era (Deemed to be University), India, and a B.Sc. in Biotechnology from Vidyasagar University, India. He is an esteemed alumnus researcher of the ICAR-IIHR, India. Dr. Mitra further expanded his academic horizons through a virtual doctoral internship at the Agroenvironmental and Health Biotechnology Laboratory, Universidad de Santander, Colombia. His scholarly endeavors are reflected in numerous publications, as well as contributions to books (publishers: Springer Nature, Apple Academic Press, Scrivener Publishing, Scholar's Press, LAP, IGI Global Scientific Publishing, Bentham Science, Cambridge Scholars and CABI), book chapters, newsletters, research technical bulletins, extended summaries, technologies, e-publications, and popular articles in leading magazines and journals. Dr. Mitra has been appointed to various editorial roles, including editor, academic editor, associate editor, review editor, and editorial board member for over 30 reputable national and international journals. Additionally, he has contributed as a Special Issue lead editor, guest editor, guest editor assistant, and topic coordinator, and has provided reviews for over 80 journals.

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Marika Pellegrini is currently working as an Agricultural Microbiology Researcher (RTD-b) at the University of L'Aquila and has been Head of the Agro-Environmental Microbiology Laboratory (Environmental Sciences Section, Dept. MeSVA, L'Aquila, Italy) since 2023. She graduated in Biology at the University of L'Aquila (Italy, 2014). She completed a PhD in Food Sciences at the University of Teramo, performing 6 months of research experience at the IPOA laboratory of the University of Miguel Hernández de Elche (Spain). She received her PhD with honours and the Doctor Europaeus certification in 2018. Her main research projects are on the selection, characterization, and exploitation of beneficial bacteria for sustainable production (agriculture, biopolymers, and bioremediation) and molecular characterization of environmental biomes. She has published 54 scientific articles in several indexed international journals, in which she appears as the first author or as the corresponding author. She is the coordinator of several national scientific projects and supervises the laboratory activities of research fellows and residents. She has participated in several projects dealing with the geomicrobiological characterization of mineral deposits and the application of microbial-based formulations to produce or recover biominerals. Her main activities concern the isolation, characterization, and application of useful strains for biotechnological applications and sustainable agriculture. She is currently involved in several national and international projects.

Leonard Koolman

Leonard Koolman is an Assistant Lecturer in Biotechnology and Molecular Microbiology at Technological University Dublin (TU Dublin), where he specializes in microbial genomics, molecular diagnostics, and bioinformatics. With a Ph.D. in Microbiology from University College Dublin, his research resides at the vital intersection of food safety, environmental health, and clinical microbiology. Dr. Koolman's work focuses on identifying and mitigating high-risk pathogens across the global food chain. His extensive portfolio includes food safety interventions, antimicrobial resistance (AMR), global health, and One Health and Climate Change. At TU Dublin, Dr. Koolman serves on the School Research Committee, contributing to strategic mentoring and research policy. He has a strong track record of securing research funding, including the SMARt project to mitigate pesticide resistance and the START Fund Award for innovative poultry biotechnology. An active member of the American Society for Microbiology (ASM) and the Klebnet-GSP Epidemiology Working Group, he has published over 25 peer-reviewed articles in high-impact journals such as *Food Control* and *Frontiers in Microbiology*. His work continues to advance sustainable microbial solutions for global agricultural and public health challenges.

Preface

Soil microorganisms—including bacteria, fungi, protozoa, and archaea, collectively referred to as the soil microbiome—are essential for plant health and crop productivity. This Reprint, "Harnessing of Soil Microbiome for Sustainable Agriculture," was developed to bridge the gap between fundamental microbial research and practical, sustainable farming solutions. Our primary motivation is the urgent need to transition away from the intensive use of synthetic chemicals, which have historically resulted in soil degradation and environmental imbalances.

The scope of this work encompasses the multifaceted interactions between plants and the soil microbiome, including bacteria, fungi, archaea, and protozoa. Throughout these pages, we explore the aim and purpose of integrating biological agents—such as biofertilizers, microbial inoculants, and biopesticides—into standard agricultural workflows. By shifting the focus toward diverse microbial communities and metagenomic insights, this collection highlights how we can enhance nutrient availability, bolster crop resilience against climate-driven stressors, and sequester atmospheric carbon.

Furthermore, this Reprint addresses the critical role of microbial bioremediation in restoring health to contaminated lands, demonstrating that microorganisms are essential partners in both production and protection.

This work is addressed to a broad audience, including soil scientists, biotechnologists, agronomists, and environmental policymakers seeking evidence-based strategies for food security. We hope that the findings presented here will inspire further innovation in microbial ecology and empower the agricultural community to foster a more harmonious relationship with the Earth's most vital hidden resource.

Debasis Mitra, Marika Pellegrini, and Leonard Koolman
Guest Editors

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Editorial

Editorial: Harnessing of Soil Microbiome for Sustainable Agriculture

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This Special Issue brings together a diverse collection of contributions that highlight the soil microbiome, including bacteria, fungi, and other microorganisms, which play a crucial role in enhancing crop productivity and nutrient absorption while minimizing dependence on synthetic chemicals. Through the application of biofertilizers and microbial biopesticides, agricultural practitioners can achieve carbon sequestration, remediate contaminated soils, and enhance their resilience to climate change. Continued research in metagenomics and biotechnology is vital for harnessing these diverse microbial communities to ensure long-term food security and the sustainability of ecosystem health. The research conducted by Zanilov et al. [1] demonstrates that specific thermophilic microbial strains, including cellulolytic organisms, nitrogen-fixing bacteria, and nitrifiers, significantly contribute to the acceleration of organic degradation, humification, and nitrogen accumulation during the composting of poultry manure. These microbial strains enhance composting efficiency by increasing small fractions by approximately 19%, elevating humic acid levels through the activity of nitrifiers by 15.5%, and substantially augmenting nitrogen content via cellulolytic and nitrogen-fixing bacteria. The findings indicate that these targeted bacterial strains facilitate the formation of smaller compost fractions and improve bioreclamation properties. This suggests that monitoring based on compost fractions is a promising strategy for optimizing biofermentation processes. Conversely, while organic waste has the potential to improve soil quality, the study by Alija-Novo et al. [2] indicates that pathogens such as *Listeria monocytogenes* can persist for several months during storage and following soil application, often enduring longer in soil than anticipated. These findings highlight the critical need for pre-treatment strategies to reduce the risks of environmental contamination and the transmission of pathogens to humans and animals.

Rawat et al. [3] conducted an innovative study that established a reproducible in vitro propagation protocol for the endangered *Nardostachys jatamansi*, utilizing shoot tip explants and growth regulators. The inoculation with *Bacillus subtilis* (MTCC 8528) and *Pseudomonas corrugata* (ATCC 29736) significantly enhanced plant survival (94.6%), chlorophyll content, and growth following soil transfer. Moreover, these beneficial microbes doubled the total phenolic content, highlighting their potential to augment both the physical resilience and medicinal efficacy of this Himalayan plant. Another study by Giannakara and Koumandou [4] employs bioinformatics to identify novel lineages with potential glyphosate-degrading capabilities by analyzing the enzymes C-P lyase and glyphosate oxidoreductase (Gox). By mapping conserved residues onto three-dimensional structural

models, researchers have gained new functional insights into the mechanisms by which these enzymes degrade pesticides. This computational approach offers a crucial framework for the discovery and optimization of microbial candidates, thereby enhancing future environmental bioremediation efforts.

The review by Miloudi-Agha and Kebdani [5] examines the potential of halophilic *Bacillus* species from saline environments as sustainable alternatives to synthetic fungicides. These species function as biocontrol agents against *Fusarium* and as promoters of plant growth. Their distinctive metabolic adaptability enables them to suppress pathogens and alleviate salinity stress through the production of bioactive compounds. This dual functionality positions them as promising, eco-friendly tools for enhancing crop resilience and promoting agricultural sustainability amidst increasing environmental challenges. An intriguing article by Maake and Sibisi [6] investigates the potential of microbial antagonists as sustainable alternatives to synthetic agrochemicals for mitigating health and environmental risks. These agents are particularly promising for managing postharvest losses in Solanaceae crops. The review examines their biological modes of action and the challenges associated with their commercialization. While further research into their stability and toxicity is necessary, these agents represent a promising, eco-friendly solution for enhancing global food security. Similarly, the study by Kumar et al. [7] highlights the critical role of soil microbes, including bio-inoculants and bioremediation agents, in sustaining soil health, facilitating nutrient cycling, and enhancing plant resilience against pests and climate change. Although advancements in metagenomics have enabled the development of customized microbial solutions for the detoxification of polluted lands, their widespread application is currently constrained by environmental variability and a paucity of large-scale field evidence. A study by Mondal et al. [8] showed that while antibiotics effectively boost silkworm growth and prevent disease by reshaping the gut microbiome, their excessive use in sericulture poses a risk of triggering antimicrobial resistance. This necessitates a shift toward more prudent management and alternative growth-promoting strategies for the species. Joshi et al. [9] highlighted that the contemporary approaches to microbiome manipulation, utilizing next-generation sequencing and synthetic biology, provide profound insights into plant-microbe interactions, thereby enhancing plant growth and stress tolerance. By employing advanced methodologies such as CRISPR/Cas9 and rhizosphere engineering, researchers are able to modulate the plant microbiome to effectively safeguard crops against pathogenic invasions and increasingly severe environmental conditions. Dzvene and Chiduza [10] highlighted how the integration of biofertilizers with push–pull cropping systems optimizes plant-microbe interactions, thereby enhancing nutrient uptake and pest control. This approach offers a synergistic, agroecological strategy to improve crop resilience and environmental sustainability. The utilization of these microbial innovations is crucial for the transition to sustainable agroecology and the assurance of global food security.

This Special Issue highlights the critical role of the soil microbiome and advanced biotechnological tools in transforming global agriculture into a more resilient and sustainable system. By utilizing microbial inoculants, metagenomics, and bioengineering techniques, researchers can enhance crop productivity, remediate polluted environments, and mitigate the risks associated with pathogens and synthetic chemicals. Ultimately, these integrated microbial strategies offer a necessary framework for securing food supplies and preserving ecosystem health in the face of escalating environmental challenges.

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Review

Mechanisms Underpinning the Biocontrol Potential of Halophilic and Halotolerant *Bacillus* Species Against *Fusarium* and Other Fungal Phytopathogens: An Eco-Friendly Alternative for Sustainable Agriculture

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Abstract

Modern agriculture is increasingly challenged by fungal diseases, with phytopathogens such as *Fusarium* species causing substantial yield and quality losses in major crops globally. Although synthetic fungicides remain widely used, their intensive application raises serious concerns regarding environmental safety, human health, and the rapid emergence of resistant pathogen populations in the environment. These limitations have accelerated the search for sustainable, biologically based alternatives. In this context, *Bacillus* species isolated from saline and hypersaline habitats have emerged as a distinctive and still underexplored group of microorganisms with dual functionality as biological control agents (BCAs) and plant growth–promoting rhizobacteria (PGPRs) in salt-affected agroecosystems. Their novelty lies in their combined ability to suppress phytopathogens, enhance plant growth, and tolerate or mitigate salinity stress. Owing to their exceptional metabolic adaptability, these bacteria remain active under osmotic stress and produce a wide range of bioactive compounds that collectively contribute to their antifungal activity and improved plant performance. This review critically synthesizes advances published over the last six years (2019–2025), providing a comprehensive overview of the current understanding of the mechanisms underlying the biocontrol potential of halophilic/halotolerant *Bacillus* species against *Fusarium* spp. and other fungal phytopathogens. Particular emphasis is placed on ecological adaptations, molecular mechanisms, and the dual roles of these bacteria as BCAs and PGPR. The exploration and exploitation of saline-adapted *Bacillus* strains offer promising, eco-friendly, and cost-effective strategies for managing *Fusarium* diseases, thereby contributing to resilient and sustainable agricultural systems under increasing environmental constraints in the future.

Keywords: halophilic/halotolerant *Bacillus* species; saline and hypersaline habitats; biological control agents; plant growth–promoting rhizobacteria; *Fusarium*; phytopathogenic fungi; salinity stress; sustainable agriculture

1. Introduction

Global agriculture is currently confronted with two interconnected challenges: continuous growth of the human population and gradual reduction in cultivable land. Together, these factors pose a serious threat to global food security. Estimates suggest that by the

year 2050, the global population may reach approximately 9.7 billion, which would require an increase of roughly 70% in overall food production [1]. Meeting this demand is only possible through sustainable agricultural practices that can perform well under environmental stress. These stresses include abiotic factors such as drought, salinity, and temperature extremes, as well as biotic pressures caused by various pests and pathogens that drastically lower both yield and crop quality [2,3].

Among biotic stressors, fungal diseases are the most damaging. They are responsible for extensive yield loss at different stages of plant development [4]. Fungal pathogens are estimated to cause 10% and 42% yield losses in major crops every year, representing nearly 70–80% of all plant diseases reported globally [5,6]. Typical symptoms include blight, wilt, root rot, canker, and leaf spot diseases, most of which are attributed to soil-borne fungi such as *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Pythium*, *Botrytis*, and *Alternaria* [7]. Within this group, *Fusarium* species are particularly important because of their wide distribution, high pathogenic variability, and impact on both field crops and stored products [8,9]. More than 300 phylogenetically distinct species have been described, forming approximately 23 recognized species complexes [10,11].

Soil salinization exacerbates disease pressure by reshaping plant–pathogen–microbe interactions. Salinity weakens plant defense systems through osmotic and ionic stress, nutrient imbalance, and oxidative damage, thereby increasing host susceptibility to fungal infections. Simultaneously, saline conditions can favor the emergence of halotolerant or opportunistic fungal pathogens, creating a "perfect storm" of compromised plant resistance coupled with persistent or stress-adapted disease pressure. This dual effect makes disease management in salt-affected soils particularly challenging and limits the effectiveness of conventional control strategies [12–14].

Traditionally, chemical fungicides have been used to manage these pathogens. Although they are generally effective, their intensive use has raised serious environmental and health concerns, in addition to the growing problem of resistant fungal strains in the field. However, their performance is often limited in saline and arid regions [15–17]. Consequently, the search for safer and more sustainable solutions has intensified, with biological control emerging as a promising alternative [18,19].

Biological control strategies employ beneficial microorganisms to reduce pathogen impact. These organisms act directly through antibiosis, mycoparasitism, or nutrient competition, and indirectly by triggering systemic resistance in plants. Many biological control agents BCAs also secrete bioactive compounds, such as antibiotics, hydrolytic enzymes, siderophores, and volatile organic compounds (VOCs), which strengthen their antifungal potential [20].

Over the past few decades, several bacterial species have been recognized as effective BCAs that can limit the development of numerous fungal diseases in plants. These beneficial bacteria act through various complementary mechanisms, including the production of hydrolytic enzymes that degrade fungal cell walls, sequestration of iron through siderophores, and the emission of VOCs. They may also trigger defense responses in plants, compete for nutrients and space in the rhizosphere, and influence the composition of the surrounding microbial community [21].

Microorganisms isolated from extreme habitats have recently attracted increasing attention. Saline and hypersaline environments, which are defined by their unique physicochemical characteristics and microbial diversity, often contain microorganisms that are well adapted to stress conditions [22]. Among them, halophilic/halotolerant bacteria exhibit remarkable physiological flexibility, allowing them to maintain their antagonistic activity even under high osmotic pressure. This resilience makes them particularly interesting

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for applications in biotechnology and agriculture, especially for the biological control of phytopathogens and stimulation of plant growth [23].

Several bacterial genera from such environments, including *Bacillus*, *Halomonas*, *Paenibacillus*, *Streptomyces*, and *Pseudomonas*, have demonstrated strong antifungal and biocontrol properties [24,25]. Within this group, *Bacillus* species have received the greatest attention because of their ecological adaptability, ability to form resistant endospores, and capacity to synthesize a wide array of antimicrobial metabolites. Their rapid growth, tolerance to environmental fluctuations, and broad-spectrum antagonistic effects make them suitable candidates for integration into sustainable agricultural systems [26].

Among bacterial genera, *Bacillus* has been one of the most intensively studied for its biological control potential [27]. These bacteria employ several complementary mechanisms to limit phytopathogen growth. They synthesize secondary metabolites, including antibiotics and LPs, which inhibit spore germination, lyse fungal hyphae, and disrupt pathogen communication (quorum quenching). In addition, they compete efficiently for nutrients and niches in the rhizosphere and can enhance plant resistance by activating of systemic defense [28–30]. Recent studies have highlighted that halophilic/halotolerant *Bacillus* strains exhibit particularly strong antifungal activity against *Fusarium* species, reinforcing their potential role in developing sustainable agricultural solutions [31,32].

The multifunctional role of halophilic/halotolerant *Bacillus* spp. is summarized in the graphical abstract, illustrating their ability to produce antifungal metabolites and enzymes, protect the rhizosphere, and promote plant growth under saline conditions. The use of halophilic/halotolerant bacteria in agrobiotechnology has been extensively reported in book chapters, research articles, and review papers. Tables 1 and 2 summarize recent research (2019–2025) on the applications of halophilic/halotolerant bacteria, with particular emphasis on *Bacillus* spp., for the biocontrol of fungal pathogens. This review focuses on the ecological functions, molecular and biochemical bases of antifungal activity, and importance of saline and hypersaline habitats as largely untapped reservoirs of stress-tolerant biocontrol strains, contributing to sustainable and environmentally friendly agricultural strategies.

2. Mechanisms Involved by Halophilic/Halotolerant *Bacillus* spp. in the Biocontrol of *Fusarium* and Other Fungal Plant Pathogens

Biological control has become one of the most promising and sustainable approaches for combating plant diseases, offering clear environmental and economic advantages over chemical-based management [33]. In recent years, halophilic/halotolerant bacteria, especially *Bacillus* species isolated from saline or hypersaline habitats, have drawn increasing scientific interest because of their strong biotechnological potential. These extremophilic microorganisms are not only well adapted to osmotic stress but also demonstrate powerful antifungal effects against *Fusarium* and other major phytopathogenic fungi [34,35].

Among the bacterial groups used as BCA, *Bacillus* is particularly notable for its ecological flexibility and consistent antagonistic performance against plant pathogens. Members of this genus are Gram-positive, spore-forming bacteria with wide genetic and phenotypic diversity, found in almost every type of environment [36]. Many *Bacillus* species are recognized as plant growth-promoting bacteria (PGPB) and efficient BCAs [37], and their success in agricultural settings is linked to several adaptive traits, including strong stress tolerance, the ability to form resistant endospores, rapid colonization of plant roots, and synthesis of numerous bioactive secondary metabolites [38].

The antifungal potential of *Bacillus* species results from a combination of complementary mechanisms. As summarized in Table 1, these bacteria produce a wide range of secondary metabolites, including LPs, VOCs, and other antifungal molecules that in-

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terfere with fungal development by disrupting hyphal structures and inhibiting spore germination [39–41]. In addition, *Bacillus* species release hydrolytic enzymes, including chitinases, β-glucanases, and proteases, which degrade fungal cell wall components and further contribute to pathogen inhibition.

Such multiple and often complementary actions explain why halophilic/halotolerant *Bacillus* strains can suppress a wide spectrum of fungal pathogens [42]. The combined effects of stress tolerance, diverse metabolite production, and enzymatic activity render these bacteria particularly valuable as eco-friendly BCA for crops cultivated in saline or degraded soils. The following sections discuss in detail the principal antifungal mechanisms identified in these bacteria and illustrate their efficiency against *Fusarium* and other important phytopathogenic fungi.

Table 1. Summary of recent studies (2019–2025) on antifungal metabolites produced by halophilic/halotolerant *Bacillus* Species, highlighting their biocontrol potential against fungal plant pathogens.

Antifungal Metabolite(s)/Traits	Bacterial Strain	Saline Habitat/Origin	Target Fungal Pathogens	Disease/Crop	References
Siderophores	*Bacillus subtilis*	Dead Sea, Jordan	*Fusarium culmorum*	Crown rot/durum wheat	[43]
Antibiotics	*B. subtilis* D6A & Dar	Salt deserts, Iran	*F. oxysporum*, *Aspergillus flavus*, *Botrytis cinerea*	Postharvest and soilborne diseases	[44]
Protease, cellulase	*Bacillus* spp.	Sabkha and Chott, NW Algeria	*F. oxysporum*, *F. verticillioides*, *Phytophthora capsici*, *B. cinerea*	Vegetable crops	[45]
Siderophores, HCN, antibiotics, protease	*B. halotolerans* BFOA1–4	Coastal saline depressions, Mediterranean Sea	*F. oxysporum* f. sp. *albedinis*	Bayoud disease/date palm	[46]
Siderophores, VOCs (acetoin, 2,3-butanediol), EPS	*B. velezensis* XT1	Saline habitat, Spain	*Alternaria alternata* and *Fusarium* spp.	Tomato, cucumber	[47]
Chitinase	*B. licheniformis* J24	Salt lake, Tunisia	*Fusarium* spp., *Sclerotinia* spp.	Corn	[48]
Protease, cellulase	*B. siamensis* S1-20	Aral Sea, Uzbekistan	*Fusarium* spp., *Verticillium dahliae*	Potato, cotton	[49]
Siderophores, hydrolytic enzymes	*Bacillus* spp.	Salt pans, Formentera, Spain	*Macrophomina phaseolina*	Charcoal rot	[50]
VOCs (acetoin, acetic acid, 2,3-butanediol, sulfur compounds)	*B. atrophaeus* L193; *B. velezensis* XT1	Saline and hypersaline environments	*F. oxysporum*, *F. solani*	Vine, potato, peach	[51]
Cyclic lipopeptides (surfactin, fengycin)	*B. halotolerans* KKD1	Qinghai–Tibet Plateau	*F. graminearum*	Wheat, barley, maize	[52]
Siderophores; amylase, lipase, protease, glucanases	*Bacillus* spp.	Salt marshes, Goa, India	*Fusarium* spp., *Rhizoctonia solani*	Chili	[31]
Cyclic lipopeptides (mycosubtilin, surfactin, bacillaene, bacillibactin)	*B. subtilis* subsp. *spizizenii* MC6B-22	Marine biofilm	*Fusarium* spp., *Colletotrichum* spp.	Not specified	[53]
Hydrolytic enzymes	*Bacillus* sp. QSLA1	Qarun Lake, Egypt	*F. oxysporum* f. sp. *lycopersici*	Tomato	[54]
Glucanase	Marine *B. subtilis* BS90	Gulf of Khambhat, India	*F. oxysporum* f. sp. *ciceris*	Chickpea	[32]

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Table 1. *Cont.*

Antifungal Metabolite(s)/Traits	Bacterial Strain	Saline Habitat/Origin	Target Fungal Pathogens	Disease/Crop	References
Lipase, protease, cellulase	*B. zhangzhouensis*	Hypersaline Aral Sea	Multiple *Fusarium* spp., *Alternaria*, *Aspergillus* spp,	Multicrop pathogens	[55]
EPS	*B. tequilensis* TSIS01	Sambhar Lake, India	*Alternaria solani*, *Fusarium* spp., *Xanthomonas citri*	Tomato blight	[56]
Cyclic lipopeptide (surfactin)	*B. amyloliquefaciens*	Ribandar salt pans, Goa, India	*F. solani*	Soilborne disease	[57]
VOCs (DAPG, pyrrolnitrin), HCN, chitinase	*Bacillus* spp.	Lake Bogoria, Kenya	*F. solani*	Common bean	[35]

Abbreviations: VOCs, volatile organic compounds; EPS, extracellular polymeric substances; HCN, hydrogen cyanide; DAPG, 2,4-diacetylphloroglucinol.

2.1. Siderophore

Microorganisms employ low-molecular-weight metal-chelating compounds, collectively termed metallophores, to acquire essential trace metals under conditions of limited bioavailability. These compounds vary in specificity, ranging from broad-spectrum metallophores capable of binding multiple transition metals to highly specific chelators [58]. Within this broader group, siderophores represent a specialized and ecologically dominant class of metallophores with high affinity for ferric iron (Fe^{3+}) and play a central role in microbial nutrient acquisition, inter-microbial competition, and biocontrol interactions

Siderophores are small organic molecules, typically with molecular weights ranging from 500 to 1500 Da, secreted by microorganisms to cope with the limited bioavailability of iron in natural environments [59,60]. The term siderophore originates from the Greek terms *sidero* (iron) and *phore* (carrier), which aptly describes their central role in microbial iron acquisition [61,62]. To date, scientists have identified over 200 distinct siderophore structures, representing approximately 500 known compounds. They are commonly divided into four major classes: hydroxamates, catecholates (or phenolcatecholates), carboxylates, and pyoverdines, although many molecules show mixed or hybrid characteristics [63].

In *Bacillus* species, genes involved in siderophore biosynthesis and iron uptake are an important part of their adaptive machinery, particularly in nutrient-poor or saline environments. For instance, *Bacillus subtilis* produces a catecholate-type siderophore known as bacillibactin, the synthesis of which is closely linked to biofilm formation. The biofilm matrix appears to enhance iron acquisition through siderophores, thereby strengthening bacterial persistence and competition [64]. This interaction between iron metabolism and biofilm development contributes significantly to the biocontrol potential of *Bacillus*, enabling the bacteria to compete effectively with phytopathogens for iron, inhibit their growth, and improve plant health [65,66]. The genetic variation and regulatory control of siderophore-related clusters further support the ecological fitness and antagonistic performance of *Bacillus* spp. through efficient nutrient sequestration [66,67].

Halophilic/halotolerant bacteria are increasingly recognized as efficient extracellular siderophore producers. In saline and iron-deficient ecosystems, these compounds not only facilitate microbial growth but also act as effective BCA by depriving fungal pathogens of iron inquired for infection and growth [68]. By modulating iron availability in the rhizosphere, siderophore-producing bacteria indirectly suppress phytopathogens via competitive exclusion, thereby promoting plant vigor and stress resilience [69].

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In halophilic/halotolerant *Bacillus* spp., siderophore-mediated iron sequestration primarily involves high-affinity chelators, such as bacillibactin, which tightly bind Fe^{3+} ions and restrict iron access to competing fungi. Iron limitation disrupts fungal metabolism and growth, induces oxidative stress through elevated reactive oxygen species and lipid peroxidation, and can lead to hyphal damage and cell death. Simultaneously, *Bacillus* spp. efficiently retrieves Fe^{3+} from siderophore complexes, maintaining a competitive advantage in iron-limited niches. In some interactions, siderophore production also suppresses specialized metabolite synthesis in antagonistic fungi, further facilitating bacterial colonization and dominance [70–72].

As documented in Table 1, several halophilic/halotolerant *Bacillus* strains exhibit strong siderophore-associated, antifungal activity. *Bacillus halotolerans* strains isolated from the Dead Sea exhibited strong antagonistic activity against *Fusarium culmorum* and *F. oxysporum* f. sp. *albedinis* [43,46], whereas Tunisian isolates suppressed *Botrytis cinerea* [73]. *B. albus* HB-17 produces both catecholate and hydroxamate siderophores [74], and *B. subtilis* RHF2, *B. amyloliquefaciens* RHF6, and *Bacillus* sp. RHFS10, isolated from Spanish salt pans, exhibited broad-spectrum antifungal activity [50]. Similarly, *B. subtilis* subsp. *halotolerans* from Goa showed activity against *F. oxysporum* and *Rhizoctonia solani* [31], and *B. velezensis* XT1 from Spain exhibited siderophore-linked inhibition against *Fusarium* spp., *Alternaria alternata*, *Monilinia fructicola*, *Magnaporthe oryzae*, and *Sclerotinia sclerotiorum* [47].

In contrast, *Bacillus* siderophores function within a multifactorial antagonistic system that integrates hydrolytic enzymes, LPs, and VOCs, whereas non-*Bacillus* halophilic/halotolerant bacteria primarily rely on siderophore-mediated competition as their primary antifungal strategy. This functional divergence underscores the ecological complementarity and potential synergism among halophilic/halotolerant bacterial genera in biocontrol. Owing to their natural origin, biodegradability, and efficiency under saline stress, siderophore-producing bacteria are promising candidates for sustainable and eco-friendly crop protection and biofertilizer development [69].

Beyond microbial competition, siderophores can enhance plant iron acquisition and alleviate abiotic stress, particularly in saline or iron-deficient soils, by solubilizing Fe^{3+} and modulating the expression of plant genes involved in iron homeostasis [75,76]. Additionally, siderophores can elicit induced systemic resistance (ISR), priming plants to respond more effectively to pathogen attacks, even in the absence of direct microbial contact [77,78]. However, most current studies have been limited to in vitro or greenhouse experiments, with few validated under field conditions where salinity, pH, and temperature fluctuate considerably [79]. The dual role of siderophores in regulating plant iron metabolism and activating immune responses highlights their untapped potential for sustainable agriculture. Integrating siderophore-producing strains with ISR inducers or micronutrient amendments could be a powerful approach to enhance disease suppression, nutrient efficiency, and stress resilience. However, more mechanistic and field-based studies are required to fully harness this potential [77,79].

Beyond their established role in plant–microbe interactions and biological control, metallophores, particularly siderophores, are increasingly recognized for their significance in human and environmental health. These low-molecular-weight chelating compounds exhibit a high affinity for iron and a wide range of metal ions, including cadmium, lead, mercury, and arsenic. Consequently, siderophores have emerged as promising tools for the bioremediation of metal-contaminated water and soil, as they facilitate metal mobilization, sequestration, and removal. Their biological origin, biodegradability, and effectiveness under diverse and extreme physicochemical conditions make them attractive, eco-friendly alternatives to conventional synthetic chelating agents, supporting their potential application in sustainable environmental remediation strategies [80].

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In addition to their environmental applications, bacterial metallophores have gained increasing attention in biomedical research as promising antimicrobial development targets. Metallophore-mediated metal acquisition is essential for bacterial survival and virulence, particularly in pathogenic microorganisms exposed to host-imposed metal deficiencies. Consequently, disrupting metallophore biosynthesis, transport, or uptake pathways can severely impair pathogen fitness, offering a novel strategy for developing new antibiotic classes. Targeting these systems may also reduce the selective pressure associated with conventional antibiotics and help address the growing challenge of antimicrobial resistance (AMR) [81]. Collectively, these findings highlight that siderophores and related metallophores are not only central to microbial ecology and plant disease management but also hold substantial potential for applications in bioremediation, public health, and next-generation antimicrobial therapies, reinforcing their relevance beyond agricultural systems.

2.2. Antibiotics

The term *antibiosis*, derived from the Greek *anti* (against) and *bios* (life), describes an interaction in which one microorganism inhibits or kills another by producing bioactive compounds. Within the framework of biological control, this phenomenon typically involves the secretion of antibiotics that interfere with vital metabolic pathways in phytopathogens, thereby restraining their growth and reproduction [82,83]. These antibiotics are generally low-molecular-weight secondary metabolites that are synthesized during the stationary phase of microbial growth. Although not essential for the basic metabolism of the producer, they provide a strong ecological advantage by helping the organism compete in complex microbial environments [84]. Actinomycetes are estimated to be responsible for approximately 85% of known antibiotics, while fungi and other bacteria account for approximately11% and 4%, respectively [85].

Since the mid-20th century, microorganisms capable of producing antibiotics have been extensively studied as potential BCA. Their ability to inhibit a wide range of plant pathogens arises from mechanisms such as membrane disruption, interference with metabolic processes, and cell lysis [86]. Among these, *Bacillus* species have been particularly well-characterized. For example, *Bacillus velezensis* has multiple biosynthetic gene clusters that encode strain-specific antifungal compounds [87].

In recent years, halophilic/halotolerant *Bacillus* strains have attracted increasing attention because of their ability to produce antibiotics under saline and osmotic stress conditions. Representative examples are provided in Table 1; strains such as *B. halotolerans* (BFOA1–BFOA4) and isolates from Iranian salt deserts exhibit pronounced inhibitory effects against *Fusarium*, *Aspergillus flavus*, and *Botrytis cinerea* through the secretion of antibiotics [44,46]. Likewise, *B. amyloliquefaciens* and *B. halotolerans* QTH8 have been shown to act as antifungal agents and as plant growth promoters [88,89]. *Bacillus safensis* isolated from saline Indian soils also demonstrated strong antifungal activity against *Sclerotium oryzae* and *Rhizoctonia solani*, the causal pathogens of rice sheath blight [90].

Accumulating evidence indicates that antibiotic-producing microbes, particularly *Bacillus* species, play a central role in suppressing plant pathogens by releasing diverse antimicrobial peptides and secondary metabolites [91]. However, translating strong in vitro inhibition into consistent in planta efficacy remains challenging. Environmental factors, such as compound instability, microbial competition, and soil salinity, can reduce the effectiveness of antibiotics under field conditions. Additionally, methodological variability across studies complicates the comparison and evaluation of these compounds [92].

Recent advances in analytical and molecular techniques, including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and genome mining, have improved metabolite profiling and may enable more standardized

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assessments of biocontrol efficacy [93]. Future research should emphasize quantitative, field-validated strategies to identify antibiotic systems that are both potent and environmentally stable for sustainable agricultural applications [91,92].

One growing concern in antibiosis-based biocontrol is the possible emergence of antibiotic resistance genes (ARGs) in soil microbial communities. Prolonged exposure to antibiotic-producing agents can create selective pressure that favors resistant populations of bacteria. Yet, metagenomic studies indicate that some integrated biocontrol formulations, especially those combining antibiotics with micronutrients such as Mg^{2+} or with metabolites that trigger induced systemic resistance, may reduce the abundance of ARGs in treated plants [94]. The likelihood of resistance emergence depends largely on the agent's mechanism of action. Microbial strains that combine antibiosis with nutrient competition, spatial exclusion, and activation of plant defenses are less prone to drive resistance than those that rely solely on antibiotic production [95,96].

Integrated management approaches are strongly recommended to mitigate these risks. These include deploying multi-strain consortia with complementary mechanisms, alternating BCA with distinct antimicrobial spectra, and optimizing agronomic practices to reduce pathogen pressure. The application of BCA at sublethal or threshold-based concentrations may also limit selective pressure without compromising disease suppression [95,96]. Similarly, threshold-based application strategies may help limit selective pressure when efficacy is maintained [94]. Moreover, because microbial biocontrol typically involves multiple mechanisms, including antibiosis, enzyme secretion, and induced resistance, it presents a much lower risk of resistance evolution than conventional single-target chemical pesticides [97].

2.3. Lipopeptide Biosurfactants

Lipopeptides (LPs) are a class of low-molecular-weight biosurfactants composed of fatty acids linked to short peptide chains, usually containing several D-amino acids. These amphiphilic molecules are well known for their ability to reduce surface and interfacial tension, as well as for their broad biological activities, including antimicrobial, cytotoxic, and surfactant properties [98,99]. Their amphiphilic structure, comprising a hydrophilic peptide moiety (often cyclic) covalently linked to a hydrophobic fatty acid chain, enables them to reduce both surface and interfacial tension and to interact efficiently with microbial membranes [57,100–102].

Cyclic lipopeptides (CLPs), in particular, are characterized by a ring-shaped oligopeptide (typically 7–10 residues) joined to a lipid tail of 10–19 carbon atoms, resulting in wide structural and functional diversity [101,103].

Most of the currently described LPs belong to well-known families, such as surfactins, iturins, fengycins, and lichenysins. However, newer compounds such as rhodoheptins and selidamides have further expanded the diversity of this group [101,104,105]. Members of the genus *Bacillus*, notably *B. subtilis*, *B. licheniformis*, and *B. atrophaeus*, are the most prolific LP producers because of their efficient biosynthetic gene clusters. Even so, other bacteria, including *Pseudomonas*, *Serratia*, *Paenibacillus*, and several marine or halophilic species, have been reported to synthesize structurally similar compounds [104].

LPs are generally assembled by large multienzyme complexes, known as nonribosomal peptide synthetases (NRPSs) [106]. Once secreted, these compounds contribute to microbial competitiveness via antagonism, biofilm formation, and surface motility. Within agricultural systems, LP-type biosurfactants act as both antifungal agents and inducers of plant defense. This dual action, which combines direct pathogen inhibition with the stimulation of systemic resistance, makes them attractive candidates for sustainable plant protection strategies [104,107]. Their antifungal mechanisms involve membrane perme-

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abilization, disruption of cell wall synthesis, and induction of oxidative stress, ultimately leading to the death of fungal cells [108]. In parallel, LPs can elicit plant immune responses, resulting in long-term protection [109].

Nevertheless, the reported minimum inhibitory concentration (MIC) and half-maximal inhibitory concentration (IC_{50}) values often vary considerably between studies due to differences in assay design (e.g., agar diffusion versus microdilution), biosurfactant purity, and the fungal species tested. Therefore, establishing standardized testing conditions is essential for meaningful cross-comparisons of LP efficacy.

Numerous studies have highlighted the antifungal potency of *Bacillus*-derived LPs. For instance, *B. subtilis* DHA41 inhibited *Fusarium oxysporum* f. sp. *niveum* by 86.4% at 100 μg/mL, mainly through the production of fengycin and iturin, respectively [110]. Similarly, *B. subtilis* SPB1 displayed MIC and IC_{50} values of 0.04 mg/mL and 0.012 mg/mL, respectively, against *Rhizoctonia bataticola*, and 4 mg/mL and 0.25 mg/mL against *R. solani*, respectively [111]. Other studies have reported the complete inhibition of *F. solani* by *B. subtilis* biosurfactants at 3 mg/mL, comparable to the chemical fungicide hymexazol [112]. Other notable examples include LPs from *B. methylotrophicus* XT1 against *Botrytis cinerea* [113], mycosubtilin, surfactin, and fengycin from *B. subtilis* against *Venturia inaequalis* [114], and iturin A from *B. velezensis* 11-5 against *Magnaporthe oryzae* [115].

MIC values ranging from 58 to 140 μg/mL have also been reported for *B. amyloliquefaciens* W0101 against *Sclerotinia sclerotiorum* and *F. oxysporum* [116], and approximately 0.8 mg/mL for *Bacillus* ZK-9 against *F. graminearum* [117]. Similarly, surfactin purified from *B. amyloliquefaciens* SK27, isolated from the Ribandar saltpans, inhibited *F. solani* and *Macrophomina phaseolina* at 3 and 1 mg/mL, respectively [57].

Despite these promising in vitro findings, relatively few studies have evaluated the effects of these compounds in greenhouses or fields. Environmental factors, such as soil adsorption, UV degradation, and plant metabolites, often limit the persistence of LPs. Combinations of fengycins and iturins have shown greater stability under field conditions than surfactin alone, suggesting that mixed LP consortia could provide more consistent protection. Interestingly, biosurfactant production in *Bacillus* tends to peak during the stationary growth phase (approximately 84 h), coinciding with sporulation [53]. Surfactin typically accumulates earlier, fengycin peaks mid-phase, while iturins dominate the later stages [118]. Understanding this temporal production profile is essential for optimizing fermentation and scaling up biosurfactant yields.

Salinity appears to play a key regulatory role in LP biosynthesis. Several studies have reported that halophilic/halotolerant *Bacillus* strains increase lipopeptide output under saline or osmotic stress, indicating that salt stress can act as a metabolic trigger rather than an inhibitor [119]. The evidence compiled in Table 1 supports this trend, with multiple saline-adapted *Bacillus* isolates exhibiting enhanced antifungal activity linked to LP production. For instance, *B. paralicheniformis* F47 from an Algerian hypersaline lake harbors NRPS gene clusters encoding surfactin, fengycin, and lichenysin [120]. *B. subtilis* SR146 from Tunisian salty soil produces the antifungal fengycin [121], whereas *B. velezensis* FMH2 and *B. subtilis* subsp. *spizizenii* FMH45, both from the Sfax solar saltern, effectively protected tomato fruits against *Botrytis cinerea* [46]. Similarly, *B. halotolerans* KKD1 inhibited *F. graminearum* through lipopeptide production [57], and *B. halotolerans* Q2H2 synthesizes surfactin, fengycin, bacillaene, and subtilosin A, showing antagonism against *Fusarium* species infecting potatoes [122].

Comparative analyses indicate that halophilic/halotolerant *Bacillus* isolates frequently produce higher lipopeptide titers under osmotic pressure, although maintaining high-salinity fermentations at an industrial scale remains economically demanding. Exploring low-sodium or co-culture systems that mimic saline stress could therefore enhance pro-

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duction efficiency. Other halophilic *Bacillus* species such as *B. tequilensis*, *B. velezensis*, *B. altitudinis*, and *B. cabrialesii*, have also been described in saline habitats, showing broad antifungal activity against *Fusarium*, *Helminthosporium*, *Colletotrichum*, *Botrytis*, and *Sclerotinia* [123–126].

Marine-derived *Bacillus* spp. also demonstrates potent antifungal potential, including *Bacillus* sp. CS30 from deep-sea seeps inhibiting *Magnaporthe grisea* [127], and *B. amyloliquefaciens* HY2–1 reduces citrus green mold caused by *Penicillium digitatum* while triggering host defense responses [128]. Similarly, *B. subtilis* RH5 from saline soils in India suppressed *Rhizoctonia solani* through the production of surfactin and bacilysin [129], whereas *B. subtilis* subsp. *spizizenii* MC6B-22 was reported to produce LPs that are highly effective against *Colletotrichum gloeosporioides* and multiple *Fusarium* species [53].

Lipopeptide production and antifungal activity are not exclusive to *Bacillus*. *Paenibacillus polymyxa* 7F1 synthesizes surfactin and iturins with activity against *Fusarium* spp. [130], while *Virgibacillus massiliensis* SM-23, isolated from Sebkha El-Meleh (Tunisia), produces locillomycin variants and bacillibactin with inhibitory effects on *Fusarium* species [131]. Yet, field studies comparing the persistence, spectrum, and phytocompatibility of non-*Bacillus* LPs are still rare. *Pseudomonas*-derived viscosins or amphisin analogs, for instance, exhibit faster degradation but greater biofilm stimulation than *Bacillus* fengycins, indicating that mechanistic complementarity, not substitution, should guide their integration into biocontrol formulations.

2.4. Volatile Organic Compounds (VOC$_S$)

VOCs are small, easily evaporated molecules produced by microorganisms that can diffuse through air and soil, enabling long-distance interactions with other organisms [132]. When emitted by microbes, these compounds are termed microbial VOCs (MVOCs). They possess broad-spectrum biological activity owing to their ability to permeate cell membranes and efficiently disperse within various environmental niches [133].

The diffusion and bioavailability of VOCs in soil are largely shaped by the physical and biological properties of soil. Factors such as texture, organic matter content, and microbial activity interact with environmental variables such as temperature and moisture, to determine how these compounds diffuse and remain available [134,135]. The presence of plant roots or soil amendments, such as biochar, can further alter VOC distribution by modifying porosity and adsorption capacity. Microorganisms act as both emitters and consumers of VOCs; when microbial diversity declines, overall VOC emissions may increase, but the chemical variety of compounds tends to decrease [134,136]. Soil chemistry and physicochemical structure also govern VOC behavior, influencing volatilization rates, diffusion through pores, and biodegradation potential of VOCs.

VOCs released from decomposing litter or root exudates can travel through microsites in the soil matrix, reshaping local microbial assemblages, modifying carbon turnover, and attracting beneficial rhizobacteria to the root zone [137]. Therefore, understanding these diffusion patterns is essential for assessing how VOCs function and persist in real soil ecosystems rather than in controlled laboratory conditions.

In addition to their ecological mobility, VOCs are increasingly recognized as key mediators of plant–microbe communication. Both plants and soil microbes can modulate their volatile profiles in response to environmental stress or signaling cues [138]. Notably, many microbial VOCs can activate induced systemic resistance (ISR) in plants, triggering internal defense pathways that strengthen resistance against fungal and bacterial pathogens. This mechanism has been linked to effective protection against several diseases, including tomato wilt [139].

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The first clear evidence of VOC-mediated antagonism was provided by Fernando et al. [140], who demonstrated that certain microbial volatiles could suppress pathogen growth without direct contact. Since then, a variety of compounds such as 2-heptanone, 3-methyl-1-butanol, 2-ethylhexanol, isovaleric acid, and isovaleric aldehyde produced by *Bacillus* spp. have been shown to efficiently inhibit *Phytophthora capsici* growth [141]. Remarkably, these volatiles are effective even at low concentrations, underscoring their potential as eco-friendly tools for biological disease management [142].

Recently, halophilic/halotolerant bacteria from saline and hypersaline environments have gained attention as novel producers of VOCs, highlighting these extremophiles as an underexplored reservoir of bioactive volatiles adapted to harsh conditions. As outlined in Table 1, several *Bacillus* spp. isolated from saline habitats produce antifungal VOCs with broad inhibitory spectra. *Bacillus velezensis* XT1 from Spanish saline soils emits acetoin and 2,3-butanediol, which suppresses *Alternaria alternata*, *Fusarium oxysporum*, *Monilinia fructicola*, and *Sclerotinia sclerotiorum* [47]. Similarly, *Bacillus atrophaeus* L193 and *Bacillus velezensis* XT1 have been shown to emit volatile molecules such as acetic acid, dimethyl disulfide, and isopentanol, which display strong antifungal properties [51]. In the same way, *Bacillus amyloliquefaciens* strains isolated from the Dead Sea have demonstrated notable antagonistic activity against *Penicillium digitatum*, the pathogen responsible for citrus green rot [143].

Moreover, *Bacillus* strain CZ-6, obtained from saline soils, produces 2-heptanone and 2-nonanone, which are key volatiles with broad-spectrum antifungal efficacy against pathogens such as *Bipolaris sorokiniana* and *Botryosphaeria dothidea* and has been associated with effective disease suppression in winter jujube (*Ziziphus jujuba* "Dongzao") [144]. Furthermore, *Bacillus halotolerans* NYG5 exhibited antifungal and antibacterial activities against *Macrophomina phaseolina* and *Rhizoctonia solani* through VOC emission [145].

Novel antifungal VOCs continue to be identified in other halophilic/halotolerant non-*Bacillus* genera. For instance, *Serratia marcescens* BKACT produces 2,4-dibromothiophenol (2,4-DBTP), which exhibits strong inhibitory activity against *Fusarium foetens* [146]. Similarly, *Stenotrophomonas rhizophila* emits β-phenylethanol and dodecanal, volatile compounds that effectively suppress *Colletotrichum gloeosporioides*, the causal agent of mango anthracnose [147]. *Paenibacillus* spp. isolated from Colombian reefs generate VOCs such as 2-furanmethanol, phenylacetonitrile, and 2,4-dimethylpentanol, which inhibit *Colletotrichum gloeosporioides*, the pathogen responsible for yam anthracnose [148].

Likewise, *Peribacillus* sp. from saline and hypersaline environments release acetoin, acetic acid, 2,3-butanediol, isopentanol, dimethyl disulfide, isopentyl, and isobutanoate, showing broad antifungal activity against multiple plant pathogenic fungi affecting vine, potato, and peach [51]. Moreover, *Brevibacterium halotolerans* JZ7 produces 2,3-butanediol and fenretinide, which significantly inhibit *Fusarium oxysporum*, causing disease in Chinese jujube [149].

Comparative assessments of VOC-mediated antagonism are hampered by methodological variability, as detection and quantification techniques, such as solid-phase microextraction (SPME), GC–MS, and compartmentalized assays, often yield inconsistent and largely qualitative results, making cross-study comparisons difficult [150–152]. Most existing studies have examined VOC activity under controlled in vitro and sealed experimental systems, which poorly represent the actual diffusion and persistence of these compounds within natural soil matrices. This methodological gap often limits the ecological validity of reported results [150–152].

A wide variety of microorganisms, including *Bacillus*, *Pseudomonas*, and *Serratia*, produce VOCs with antifungal properties and growth-promoting effects on plants. However,

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the composition and biological function of these VOC blends vary greatly among species and even among strains and are strongly shaped by environmental factors [150,151,153].

In general, halophilic/halotolerant *Bacillus* species release a smaller diversity of VOCs than *Pseudomonas* or *Serratia*, yet key metabolites such as acetoin and 2,3-butanediol display a dual role in suppressing phytopathogens while stimulating plant growth which can be especially beneficial under saline or stressful conditions [150]. Despite these promising traits, only a limited number of studies have established quantitative links between specific VOCs, pathogen inhibition rates, or the induction of systemic resistance in planta; most findings remain descriptive rather than mechanistic [150,151,153].

To enhance practical application, recent research has emphasized the need to combine quantitative VOC flux analysis, soil-based microcosm assays, and multi-omics approaches. Such integration would help clarify how abiotic variables influence VOC biosynthesis, persistence, and biocontrol [150,151]. Ultimately, this knowledge could guide the selection or even the engineering of halophilic/halotolerant *Bacillus* strains that are optimized for VOC-mediated disease control in challenging environments.

2.5. Hydrogen Cyanide (HCN)

HCN is a volatile secondary metabolite known for its potent inhibitory effects on a wide range of organisms. In biocontrol contexts, HCN serves as a microbial defense molecule that suppresses phytopathogens by targeting cytochrome c oxidase and interfering with metalloenzyme activity, thereby disrupting respiratory metabolism in sensitive organisms [154]. Cyanogenesis, the microbial synthesis of HCN has long been recognized as a trait associated with the antagonistic behavior of rhizospheric and soil-dwelling microbes [155]. Several plant-associated bacteria, including those isolated from halophytes or saline habitats, exhibit cyanogenic activity as an antifungal strategy.

The agricultural interest in HCN-producing microbes has grown steadily, given their eco-friendly nature and effectiveness as natural biopesticides [156]. Emerging studies also suggest that extremophiles, such as those from Antarctic soils, may possess the capacity to produce HCN or structurally similar antifungal compounds, warranting deeper exploration of microbial biodiversity in extreme environments [157]. These findings warrant a broader exploration of microbial biodiversity in extreme environments [158].

Within saline and hypersaline settings, halophilic/halotolerant bacteria have evolved adaptive mechanisms including the biosynthesis of antifungal metabolites such as HCN [159]. As indicated in Table 1, although *Bacillus* species are not typically regarded as major HCN producers, several halophilic/halotolerant strains have been reported to exhibit HCN-mediated antagonistic activities. For example, *Bacillus halotolerans* isolates (BFOA1–BFOA4) synthesize HCN and effectively inhibit multiple *Fusarium* species, including *F. oxysporum*, *F. solani*, *F. chlamydosporum*, and *F. acuminatum* [46]. Similarly, *Bacillus subtilis* RH5 from saline soils has been reported to control rice sheath blight (*Rhizoctonia solani*), partly through HCN production [129]. Other halophilic/halotolerant bacteria such as *Bacillus safensis* BKACT [74,146], *B. amyloliquefaciens*, *B. velezensis*, *B. vallismortis*, *Ochrobactrum thiophenivorans*, and *Serratia* sp. SB6 also exhibits HCN-linked antifungal activity, contributing to pathogen suppression in salt-affected agricultural soils [143,160].

While cyanogenesis in *Bacillus* is generally viewed as an accessory antifungal mechanism, it represents a core defense strategy in genera such as *Pseudomonas* and *Serratia*. Strains from saline soils belonging to these taxa show consistent HCN biosynthesis and often enhanced cyanogenic activity under high NaCl conditions, which significantly strengthens their antifungal performance [90].

The physiological effects of HCN on plants are dependent on its concentration. At elevated levels, it acts as a phytotoxin, causing tissue damage or necrosis; however, at

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sub-toxic concentrations, it can act as a signaling molecule that modulates plant stress responses, pathogen defense, and certain aspects of primary metabolism [161]. HCN and its precursors, such as cyanogen, degrade relatively quickly in soil, suggesting limited persistence in the environment, although precise safety thresholds for crops and agricultural workers have yet to be established [162].

Traditionally viewed as a BCA, HCN produced by plant growth–promoting rhizobacteria (PGPRs) may also regulate nutrient availability, particularly phosphate mobilization via metal chelation, rather than solely suppressing pathogens. Interestingly, beyond its antimicrobial function, HCN may facilitate nutrient cycling by chelating metals and enhancing phosphate solubility, thereby indirectly supporting the nutrition of plants.

To safely exploit HCN in biocontrol, its concentration and exposure must be carefully regulated to maintain beneficial levels while avoiding phytotoxicity. Possible strategies include optimizing delivery methods, selecting tolerant crop species, and employing co-treatments that stimulate detoxification pathways. Further studies are needed to define the conditions under which HCN provides optimal biocontrol and nutrient-mobilizing benefits without compromising plant health [163].

Despite its broad-spectrum antimicrobial reputation, HCN production does not always correlate with pathogen suppression or plant protection, especially under saline or stressful field conditions [163,164]. In many cases, it seems to complement rather than dominate microbial antifungal mechanisms, acting alongside more potent compounds such as LPs and hydrolytic enzymes [164]. The inconsistent results across studies are partly due to methodological differences in HCN detection, which range from qualitative colorimetric assays to semi-quantitative tests, making cross-study comparisons difficult [163].

Emerging data suggest that HCN also plays roles beyond direct antagonism. It can regulate nutrient bioavailability particularly phosphate mobilization in the rhizosphere, thereby indirectly promoting plant growth under nutrient-limited conditions [161,163]. Genetic and transcriptomic studies using mutant strains have further shown that HCN influences bacterial physiology, affecting traits such as motility, biofilm formation, and the synthesis of other antimicrobial metabolites.

These findings indicate that cyanogenesis can shape both the producing bacterium's behavior and the surrounding microbial community [165]. Future research should thus focus on quantitative HCN measurement, mutant-based functional analyses, and in situ validation to clarify its ecological and biocontrol roles, particularly under realistic saline field conditions [165].

2.6. Exopolysaccharides (EPS)

Biofilms are complex, organized microbial communities encased in a self-generated extracellular matrix largely composed of EPS [166]. This matrix allows microorganisms to anchor themselves to both living and inert surfaces, while simultaneously serving as a shield against environmental pressures such as antimicrobial agents, dehydration, and salinity stress [167].

EPS are high–molecular-weight polymers secreted by microbes in two major forms: capsular polysaccharides that remain tightly bound to the cell surface, and slime polysaccharides that are loosely attached or freely released into the surrounding medium [168]. Together with proteins, lipids, and nucleic acids, these polymers give biofilms their structural strength, maintain hydration, and help microbial communities withstand external challenges [169].

In agricultural systems, EPS-producing bacteria particularly those belonging to *Bacillus*, *Burkholderia*, and *Paenibacillus polymyxa* have demonstrated the ability to protect plants against major fungal pathogens such as *Fusarium oxysporum*, *Phytophthora* spp., and *Botrytis*

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cinerea [170]. For example, *Bacillus amyloliquefaciens* W19 improved disease suppression when applied together with organic fertilizers [171], while *B. pumilus* and *B. toyonensis* showed inhibitory activity against *Rhizoctonia solani* and *B. cinerea* [172,173].

However, most studies have been restricted to in vitro or greenhouse settings, and the consistency of EPS-based protection under fluctuating field salinity is still uncertain. Optimizing inoculum formulation and delivery methods remains essential to ensure EPS persistence and functional stability in soil.

Under saline and hypersaline conditions, biofilm formation is a vital survival strategy. Rhizobacteria rely heavily on EPS synthesis to cope with osmotic stress, drought, and variable pH [174]. The ability to form biofilms not only enhances bacterial colonization in the rhizosphere but also helps beneficial microbes outcompete pathogens and establish protective niches that limit disease development [175,176].

EPS production is therefore a defining adaptive feature of halophilic/halotolerant bacteria, linking environmental resilience with antifungal potential. The evidence compiled in Table 1 indicates that several *Bacillus* species isolated from saline habitats produce bioactive EPS with strong biocontrol activity. For example, *Bacillus velezensis* FMH2 and *B. subtilis* subsp. *spizizenii* FMH45, both isolated from the Sfax saltern in Tunisia, produced EPS that significantly reduced *Botrytis cinerea* infection in tomato plants [73]. Likewise, *B. velezensis* XT1, obtained from a Spanish saline environment, secreted EPS that inhibited *Alternaria alternata* and *Fusarium* spp., while simultaneously improving plant growth and salt tolerance in tomato and cucumber [47]. Another example is *Bacillus tequilensis* TSIS01 from the Sambhar Lake region of India, which produced EPS with strong antifungal activity against *Alternaria solani*, *Fusarium oxysporum*, and *F. solani*, as well as antibacterial action against *Xanthomonas citri*, leading to reduced blight incidence in tomato [56].

Comparative analyses show that halophilic/halotolerant *Bacillus* strains can generate large amounts of EPS even under high salinity, with some isolates producing up to 20 g/L [177,178], and maintaining growth at NaCl concentrations of 15% [177,178]. These EPS-producing halophiles also display increased tolerance to heavy metals and stronger biofilm formation, both of which support their persistence in salt-affected soils [177,179]. Despite these advances, few studies have quantitatively compared EPS yield, viscosity, ionic-binding properties, or biophysical stability between halophilic and non-halophilic *Bacillus* strains.

Available data suggest that EPS from halophilic *Bacillus* and related genera possess notable metal-binding capacities and plant growth-promoting potential under saline stress, but detailed compositional and mechanistic analyses remain limited [177–179]. This gap highlights the need for systematic, quantitative investigations to clarify how halophilic EPS differ from their non-halophilic counterparts in yield, structure, and stability.

By enabling biofilm formation, nutrient retention, and rhizosphere colonization, EPS-producing halophilic/halotolerant bacteria represent promising tools for crop protection and stress mitigation in saline and marginal soils. Their use as biofertilizers has already shown beneficial impacts on crop productivity in wheat, maize, chickpea, and sugarcane under salt stress [180,181]. A major challenge, however, lies in the variability of EPS composition across strains and environments, which complicates formulation standardization. Integrating omics-based approaches to link EPS structure with biocontrol performance could help overcome this limitation [182,183].

EPS is a core matrix component that stabilizes multicellular biofilms, supporting high local cell density, coordinated secretion of hydrolytic enzymes, antibiotics, siderophores, and VOCs, and persistent root or tissue colonization. In *Bacillus* HR10, biofilm formation is linked to lipopeptide antibiotic production, which contribute to pathogen inhibition and induce systemic resistance in the host [172–184]. EPS not only protects beneficial bacteria

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but also facilitates nutrient exchange and modulates microbial community structure to favor BCA [185].

Furthermore, siderophore biosynthesis [186,187] is often linked to EPS and biofilm regulatory pathways; reduced EPS levels can lead to lower siderophore output and weaker biofilm development. Interestingly, EPS from beneficial bacteria can also inhibit pathogenic biofilms while reinforcing their own, integrating multiple mechanisms to ensure microbial resilience and disease suppression [184,186–188].

EPS-producing halophilic/halotolerant bacteria typically express several additional plant growth-promoting traits, including the production of phytohormones Indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), siderophores, and phosphate-solubilizing enzymes. Together with robust root colonization, these factors act synergistically to enhance nutrient uptake, stress tolerance, and protection against phytopathogens [189–192].

Beyond biocontrol, EPS contribute to broader ecological processes such as ionic balance regulation, improved soil water retention, and nutrient chelation, all of which strengthen plant performance under saline conditions [193]. The convergence of these two fields represents a promising but still emerging direction for sustainable crop protection, though biosafety and regulatory assessment remain key priorities before field application.

2.7. Cell Wall-Degrading Enzymes (CWDEs)/Hydrolytic Enzymes

Hydrolytic enzymes such as chitinases, glucanases, proteases, and cellulases play a crucial role in the biological control of fungi by degrading key structural components of fungal cell walls. Chitinases break down chitin, a major fungal cell wall polymer, disrupting fungal integrity and inhibiting growth, which significantly reduces fungal infection in plants.

Glucanases target β-glucans, another essential polysaccharide in fungal walls, weakening the pathogen's defense. Proteases degrade fungal proteins, contributing to cell wall breakdown and fungal cell death, while cellulases hydrolyze cellulose components, further compromising fungal structure [194,195]. These enzyme systems often act synergistically; combined activity of chitinases, glucanases, proteases, and cellulases results in severe hyphal distortion, swelling, and breakage, thereby greatly enhancing antifungal efficacy [194,195].

The main mechanism of action involves the enzymatic degradation of key polysaccharides such as chitin, cellulose, and hemicellulose major constituents of fungal cell walls. By hydrolyzing glycosidic linkages in these polymers, the enzymes compromise cell wall rigidity, interfere with hyphal growth, and inhibit germ tube formation. The result is a weakening of the pathogen's structural integrity and reduced ability to penetrate plant tissues [196–198]. Beyond their direct lytic effect, these enzymes can also elicit defense responses in plants, contributing to enhanced systemic resistance.

As CWDEs, these hydrolases are crucial components of microbial antagonism during plant colonization. Their significance becomes even more evident in saline agriculture, where halophilic/halotolerant bacteria organisms naturally adapted to high salinity and environmental stress have emerged as prolific producers of such enzymes. These microorganisms secrete a broad array of hydrolytic enzymes, including β-1,3-glucanases, chitinases, cellulases, proteases, and DNases, all exhibiting notable antifungal properties [55,199,200].

Their enzymes often possess adaptive structural traits such as higher proportions of acidic amino acid residues and flexible catalytic regions that enable efficient catalytic activity even under extreme salinity, temperature variation, and desiccation [193,201]. Consequently, they represent promising candidates for biocontrol applications in salt-affected soils, where conventional microbial agents typically lose efficacy.

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Each enzyme contributes differently to the antifungal process. Chitinases and β-glucanases target and hydrolyze the main polysaccharide backbones of fungal cell walls, whereas proteases degrade structural and functional fungal proteins associated with virulence. The coordinated activity of these enzymes produces a synergistic and multi-layered antifungal effect combining cell wall lysis, nutrient depletion, and inhibition of spore germination to provide plants with a stronger defense against pathogenic fungi.

Halophilic/halotolerant bacteria employ a variety of hydrolytic systems including cellulases, proteases, lipases, and amylases as part of their antifungal repertoire. Each enzyme class contributes to the degradation of distinct fungal cell wall components, and their overall efficacy depends on substrate specificity as well as environmental parameters such as salinity and pH [73,74,202]. Table 1 summarizes evidence that halophilic/halotolerant *Bacillus* strains isolated from saline environments produce diverse CWDEs that play a key role in their antagonistic activity against fungal phytopathogens. Their antifungal effectiveness is also shaped by their stability under stress and by synergistic interactions with other bioactive compounds, including siderophores, biosurfactants, and VOCs, which can amplify pathogen inhibition [73,74,202].

Variations in experimental methodologies spanning from in vitro antagonism assays to genomic and biochemical analyses often influence how antifungal potential is measured, making it difficult to compare findings across studies. Nonetheless, field evaluations are encouraging. Several reports indicate that both individual halophilic/halotolerant strains and microbial consortia, particularly those belonging to *Bacillus*, *Halomonas*, and *Serratia*, can effectively suppress fungal pathogens and enhance plant growth under saline stress [73,74,202,203]. Some strains are also capable of forming protective biofilms around roots, improving plant tolerance to salinity and disease.

The ultimate success of these enzyme-based biocontrol systems in agricultural practice, however, depends on their persistence in soil, compatibility with crops, and ability to maintain activity under variable field conditions [73,74,202].

2.7.1. Chitinases

Chitin, the second most abundant natural polymer after cellulose, plays a fundamental structural role in fungal cell walls and in the exoskeletons of arthropods [204]. To target this key structural component, many microorganisms synthesize chitinases (Enzyme Commission (EC) 3.2.1.14) glycosyl hydrolases ranging from 20 to 90 kilodalton (kDa) that catalyze the hydrolysis of β-1,4 glycosidic bonds in chitin, yielding N-acetylglucosamine monomers [205].

Chitinases are broadly distributed across bacteria, actinomycetes, fungi, and plants, and have attracted particular attention in agriculture for their potential as eco-friendly biocontrol tools. By selectively degrading fungal cell walls without generating toxic residues, these enzymes offer a sustainable alternative to chemical fungicides [206]. Based on their cleavage mechanisms, chitinases are categorized into four main types: endochitinases, exochitinases, β-N-acetylglucosaminidases, and chitobiases.

In recent years, halophilic/halotolerant bacteria from saline and hypersaline environments have been recognized as promising sources of chitinases with exceptional stability and biocontrol efficiency. These enzymes maintain catalytic activity under osmotic and saline stress, making them particularly suitable for managing plant diseases in salt-affected soils [207].

For instance, the marine-derived *Bacillus* isolate B26 inhibited *Fusarium solani* and *Penicillium chrysogenum* by 69% and 46.6%, respectively [208]. Similarly, *Bacillus licheniformis* J24, isolated from a Tunisian Salt Lake, exhibited strong antagonism against *Fusarium pseudograminearum* via chitinase secretion [48]. In India, *Bacillus safensis* strains produced

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high levels of chitinase that effectively suppressed *Sclerotium oryzae* and *Rhizoctonia solani*, thereby reducing rice sheath blight and related fungal diseases [74,129].

Several studies have characterized the catalytic performance and stability of bacterial chitinases under varying salinity conditions, underscoring their potential in saline agroecosystems. The chitinase from *Virgibacillus marismortui* M3-23, for example, maintains high activity and stability across a wide range of salinities, pH levels, and temperatures, showing both halo- and thermo-tolerance properties valuable for industrial and agricultural applications [209]. Similarly, *Paenibacillus pasadenensis* NCIM 5434 produces a chitinase that remains active at up to 3% NaCl, confirming its moderate halotolerance and suitability for crop protection in saline soils [210].

Enzyme stability under salt stress is a decisive factor for their biotechnological application. Chitinases from halophilic/halotolerant bacteria such as *Bacillus pumilus* MCB-7, *Streptomyces*, and *Halomonas* species retain high activity at elevated salt concentrations and temperatures, with some maintaining function up to 60 °C and at NaCl levels exceeding 10% [211–214]. Most exhibit optimal activity at moderate salinities (2–6% NaCl) while preserving function beyond this range.

This resilience is commonly attributed to structural adaptations such as enriched acidic amino acid residues and the accumulation of compatible solutes, which help stabilize protein conformation under osmotic stress [209,210]. Additional halophilic/halotolerant bacteria, including *Bacillus licheniformis* and various *Halomonas* species, have been shown to produce chitinases and other extremozymes, with enzyme activity and stability often enhanced by the production of compatible solutes under salt stress [211].

The dual capacity to tolerate high salinity and suppress fungal pathogens makes chitinases particularly promising for sustainable agriculture in marginal soils. Their application not only reduces dependence on chemical fungicides but also enhances crop resilience in challenging environments [215,216].

Numerous reports confirm that bacterial chitinases, especially those halophilic/halotolerant *Bacillus* strains, maintain strong antifungal activity under saline conditions, functioning effectively across broad temperature and pH ranges [217].

Nevertheless, much of the available data derives from in vitro assays using purified or semi-purified enzymes, often with heterogeneous substrates (e.g., colloidal vs. crystalline chitin). The absence of standardized inhibition indices and variability in assay conditions complicate direct comparisons between studies [218]. While enzyme kinetics and optimal activity parameters have been characterized for several strains, few studies have correlated these findings with field-level suppression of diseases such as *Fusarium* wilt. In planta validations therefore remain scarce [217,219].

Despite these methodological gaps, chitinase-producing *Bacillus*, *Halomonas*, and marine bacterial strains continue to represent highly promising biocontrol candidates for salinity-affected agroecosystems due to their enzyme stability and broad antifungal spectra [217]. Recent research has even explored the engineering of hybrid chitinases with enhanced antifungal potency, though these innovations are still largely restricted to laboratory-scale evaluation [219]. To fully realize the potential of bacterial chitinases in sustainable crop protection, future efforts should focus on improving formulation stability, assessing efficacy under variable field conditions, and establishing standardized activity assays [217,219].

2.7.2. Cellulases

Cellulose, a rigid and insoluble polysaccharide composed of linear β-1,4-linked glucose units, forms tightly packed crystalline microfibrils that are highly resistant to degradation. Its breakdown requires a coordinated enzyme system known as cellulase,

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which includes endo-1,4-β-glucanases (EC 3.2.1.4), exo-1,4-β-glucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) [220]. Acting synergistically, these enzymes hydrolyze β-1,4-glycosidic bonds within cellulose chains, releasing cellobiose and glucose as end products [221].

Beyond their established roles in carbon cycling and biomass turnover, cellulases have attracted increasing attention in sustainable agriculture particularly as components of biocontrol systems in salinity-affected soils, where conventional disease management strategies often fail [207,222]. A wide variety of microorganisms including bacteria, fungi, and actinobacteria exhibit cellulolytic activity and contribute to the biological suppression of phytopathogens [197].

Cellulolytic activity is consistently observed in *Bacillus* species, which can degrade plant residues and polysaccharides in fungal cell walls. For example, *Bacillus subtilis* has demonstrated cellulase-mediated antagonism against *Colletotrichum gloeosporioides*, the causative agent of anthracnose in chili [223]. Similarly, *B. subtilis* TD11 inhibited multiple fungal pathogens, including *Fusarium*, *Rhizoctonia*, *Colletotrichum*, and *Aspergillus* spp. [224]. Another notable strain, *B. velezensis* TSA32-1, produces cellulases with potent antifungal activity against *Fusarium fujikuroi*, *F. graminearum*, and *Alternaria alternata* [39].

Halophilic/halotolerant bacteria from saline and hypersaline environments have proven to be particularly reliable sources of cellulases due to their ability to maintain enzymatic activity under high salinity. This property renders them especially valuable in challenging agricultural contexts, such as salt-affected soils, where conventional microbial BCA may lose efficacy [222]. Within this group, *Bacillus* species are distinguished by their robust cellulolytic capacity, essential for degrading the cellulose-rich components of fungal cell walls [225]. For instance, *Bacillus* sp. CPO 4279 demonstrated significant antifungal activity, as evidenced by IC_{50} value [226]. Likewise, *B. amyloliquefaciens* strains isolated from the Dead Sea and Moroccan coastlines displayed both cellulase activity and effective suppression of postharvest citrus pathogens [143].

In a large-scale survey of 196 halophilic/halotolerant *Bacillus* isolates from coastal salt marshes in Goa, India, strains producing combinations of cellulases and other hydrolases were particularly effective in protecting chili plants (*Capsicum annuum* L.) from soilborne fungi, including *Fusarium oxysporum*, *F. pallidoroseum*, *Rhizoctonia solani*, and *Pythium aphanidermatum* [31]. Similarly, *Bacillus siamensis* S1-20 and *B. zhangzhouensis* from the Aral Sea exhibited broad-spectrum antifungal activity, suppressing multiple *Fusarium* species, including *F. annulatum*, *F. oxysporum*, *F. culmorum*, *F. brachygibbosum*, *F. tricinctum*, and *F. verticillioides* [49]. Halophilic non-*Bacillus* taxa, including *Halomonas* and *Virgibacillus* spp. from Algerian chotts, also display strong cellulase and xylanase activity, confirming their metabolic versatility and contribution to nutrient cycling in saline rhizospheres [45].

Quantitative comparisons across halophilic/halotolerant bacteria reveal substantial variability in cellulase production, reflecting both species-specific metabolic capacity and adaptation to salinity. *Bacillus paralicheniformis* produced 0.042 U/mL of cellulase at 15% NaCl, approximately tenfold higher than *Halomonas* spp. under similar conditions (0.004–0.042 U/mL) [211]. Remarkably, *Bacillus* sp. PM06 produced 15 ± 1.5 U/mL of cellulase at ~11.7% NaCl, representing one of the highest yields reported among halophilic/halotolerant strains [227], while *Bacillus* sp. AMNP1.17 showed its highest activity (0.076 U/mL) at ~2.9% NaCl [228], highlighting the influence of moderate salinity on enzyme induction.

In contrast, actinomycetes and true halophiles often surpassed these yields; *Nocardiopsis* sp. SSL14 achieved 1.08 U/mL at 12% NaCl [229], indicating that moderate salinity can enhance enzyme induction. The most potent cellulase producer reported among true halophiles was *Salinivibrio* sp. SP9, with 1.95 U/mL activity at 12.8% NaCl [230]. Although

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Bacillus subtilis SR60 was evaluated qualitatively, it retained cellulase activity up to ~8.8% NaCl, confirming the resilience of *Bacillus*-derived enzymes under extreme osmotic conditions [231]. Optimal activity for these cellulases typically occurs at neutral to slightly acidic pH (6.0–7.0) and moderate to high temperatures (50–70 °C) [227,232].

These quantitative insights highlight the variability and robustness of cellulase production among halophilic/halotolerant bacteria and underscore the potential of extremophilic bacteria species as dual-function agents for both biomass degradation and fungal suppression in saline agroecosystems.

Cellulase-producing halophilic/halotolerant *Bacillus* strains, such as *B. halotolerans*, *B. amyloliquefaciens*, and *B. mojavensis*, consistently demonstrate strong in vitro antifungal activity, largely due to the combined action of hydrolytic enzymes (cellulases, chitinases, proteases) and antifungal metabolites [117,122,233,234]. However, methodological heterogeneity such as variations in assay substrates, NaCl concentrations, temperature, and pH complicates direct comparisons of enzyme activity and biocontrol efficacy, as most studies rely on agar-based or reducing-sugar assays that may not accurately represent soil or rhizosphere conditions [122,232,233].

Evidence suggests that moderately halophilic/halotolerant *Bacillus* species, notably *B. halotolerans* and *B. amyloliquefaciens*, achieve an optimal balance between enzyme induction and stress resilience. These strains often outperform true halophiles in plant-associated environments due to their capacity to colonize roots, form biofilms, and maintain enzymatic activity under moderate salinity. They have demonstrated effective in planta disease suppression, including reduced postharvest decay in apple and tomato and control of *Fusarium* wilt and wheat head blight, frequently surpassing chemical fungicides in efficacy [117,122,232–234].

Genomic analyses reveal that these strains harbor diverse gene clusters for antifungal LPs (e.g., surfactin, fengycin, bacilysin) and plant growth-promoting traits, further supporting their dual-function potential [117,122]. To advance practical applications, standardized enzymatic assays and greenhouse or field validations are necessary to better correlate in vitro enzyme activity with real-world biocontrol outcomes [122,232,233].

2.7.3. Proteases

Proteases (also referred to as proteinases; EC 3.4.24) constitute a diverse class of hydrolytic enzymes that play a central role in microbial antagonism against fungal pathogens. Based on their catalytic residues, proteases are classified into four main groups: aspartic, cysteine, serine, and metalloproteases [235].

Beyond degrading structural components, proteases can inactivate extracellular virulence factors secreted by phytopathogenic fungi, reducing their infectivity and capacity to colonize plant tissues [236]. Extracellular bacterial proteases are particularly advantageous, as they retain activity across diverse environmental conditions and can degrade pathogenic proteins on plant surfaces [237].

Several microbial genera are recognized for their protease-mediated antifungal activity. For example, *Bacillus velezensis* TSA32-1 demonstrates potent activity against *Fusarium fujikuroi*, *F. graminearum*, *Alternaria alternata*, and *Pythium ultimum* via protease secretion [39]. Other *Bacillus* species, including *B. cereus* and *B. pseudomycoides*, have also exhibited antifungal efficacy against tomato pathogens [238]. A thermostable serine protease from *Bacillus licheniformis* W10 highlights the potential of proteases as heat-resistant BCA [239].

In saline and hypersaline environments, halophilic/halotolerant bacteria have evolved proteolytic systems that remain active under high-salt conditions, an important trait for disease management in salt-stressed agricultural soils [240]. Such extremozymes facilitate the degradation of fungal cell walls even under hostile environmental conditions. Protease

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activity is widespread among halophilic *Bacillus* strains, with notable examples including *B. subtilis* RH5 [129], *B. siamensis* S1-20 [49], and *B. zhangzhouensis* from the Aral Sea [55]. Similarly, *B. amyloliquefaciens* RHF6 and *B. halotolerans* RHF12, isolated from a natural salt park in Spain, demonstrated strong antagonism against *Macrophomina phaseolina*, attributed to their robust protease activity [241].

Other *B. amyloliquefaciens* strains from saline soils effectively suppressed *Botrytis cinerea* and *Colletotrichum gloeosporioides* in jujube [242]. In Tunisia, *B. velezensis* FMH2 and *B. subtilis* subsp. *spizizenii* FMH45, isolated from the Sfax saltern, exhibited high proteolytic and antifungal activity against *B. cinerea* in tomato [73]. Likewise, *Bacillus amyloliquefaciens* strains from saline soils effectively suppressed *Botrytis cinerea* and *Colletotrichum gloeosporioides* in jujube [144]. Collectively, these findings underscore the significance of proteases in degrading fungal structural proteins and spore wall components, thereby enhancing the biocontrol potential of halophilic/halotolerant *Bacillus* species in saline environments.

Although protease-mediated biocontrol shows promise in vitro and in controlled in planta systems, field-level evidence remains limited. For example, proteases from *B. amyloliquefaciens* and *Aureobasidium pullulans* can inhibit fungal pathogens and reduce disease symptoms under laboratory or postharvest conditions, with efficacy influenced by enzyme concentration and storage parameters [243]. Translation to field conditions is challenging, as environmental variability, microbial interactions, and plant physiology can affect outcomes [244,245]. This underscores the need for robust field trials to confirm the practical efficacy and reliability of protease-based biocontrol strategies.

Bacillus species, including *B. licheniformis* and *B. amyloliquefaciens*, remain promising protease sources for commercial and field applications due to the thermostability and halotolerance of their enzymes. Protease-mediated antagonism has been clearly demonstrated: purified proteases from these strains directly inhibit fungal growth, and inhibition is abolished when protease activity is blocked, confirming the central role of proteolysis.

However, quantification of enzyme kinetics often lacks standardization, with many studies relying on qualitative or semi-quantitative assays rather than robust approaches such as casein or azocasein hydrolysis, limiting inter-study comparability [239,243].

Synergistic interactions between proteases and other *CWDEs*, including chitinases and glucanases, are frequently suggested, and *Bacillus* strains often co-produce these enzymes. Nevertheless, direct experimental evidence for synergy is limited; the specific contributions and interactions of individual enzymes in pathogen suppression are not always dissected [246,247]. For example, some *Bacillus* strains exhibit strong biocontrol activity and secrete multiple enzymes, yet the specific contribution and interaction of each enzyme type in pathogen suppression are not always dissected experimentally [247], while laboratory data are promising, translating these findings to consistent field performance remains challenging, as environmental conditions can affect enzyme stability and activity. Further research is needed to optimize formulations and validate protease-based biocontrol strategies under real-world conditions [239].

2.7.4. β-Glucanases

β-Glucanases are hydrolytic enzymes that target β-glucans major structural polysaccharides of fungal cell walls. Among these, β-1,3-glucanases are the most extensively studied due to their capacity to cleave β-1,3-glycosidic linkages, while some also act on β-1,6 branches. This enzymatic degradation weakens the cell wall structure, disrupts fungal morphogenesis, and ultimately suppresses pathogen development [194,248].

Based on their mode of action, β-glucanases are divided into two types: exo-β-1,3-glucanases, which release glucose monomers from non-reducing ends, and endo-β-1,3-glucanases, which hydrolyze internal bonds to produce oligosaccharides. Both forms

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contribute to plant defense by lysing fungal pathogens and reducing their virulence. Because of this dual functionality, β-glucanase-producing bacteria are increasingly viewed as eco-friendly alternatives to chemical fungicides [249].

Several bacterial genera such as *Bacillus*, *Clostridium*, and *Chitinophaga* have been identified as efficient producers of β-glucanases with antifungal potential. For instance, *Bacillus mojavensis* inhibited the kiwifruit pathogen *Didymella glomerata* through β-1,3-glucanase secretion [250]. Likewise, *Bacillus subtilis* and *B. methylotrophicus* strains achieved more than 75% inhibition against *Alternaria triticina* and *Bipolaris sorokiniana* [251]. Marine-derived *B. subtilis* isolates from the Gulf of Khambhat (India) also demonstrated strong β-glucanase activity against *Fusarium oxysporum* f. sp. *ciceris*, highlighting the marine ecosystem as a promising source of novel antifungal enzymes [32].

In saline and hypersaline environments, halophilic/halotolerant bacteria are particularly adept at producing β-glucanases that remain active under high-salt conditions—an important advantage for use in salt-affected agricultural soils. Strains such as *Bacillus subtilis*, *B. velezensis*, and *B. paralicheniformis* exhibit strong glucanolytic activity and antifungal effects against pathogens including *Fusarium* spp. and *Colletotrichum gloeosporioides* [226,252,253]. Notably, *B. halotolerans* Q2H2 inhibited multiple phytopathogens through β-1,3-glucanase production [131]. Similarly, *Halomonas meridiana* ES021, isolated from saline ponds in Qarun Lake (Fayoum, Egypt), secreted extracellular β-1,3–1,4-glucanase with inhibitory effects against *Penicillium* spp. and *Aspergillus niger* [254]. Other halophiles, including *Virgibacillus marismortui* and *Terribacillus halophilus*, co-produced β-glucanases and chitinases, thereby broadening their antifungal activity spectrum [255].

A growing body of evidence indicates that β-glucanases often act synergistically with other lytic enzymes, such as chitinases and proteases, to enhance fungal cell wall degradation. While β-glucanases target β-glucans, chitinases hydrolyze chitin, and together they achieve more extensive cell wall breakdown than either enzyme alone. For instance, *Bacillus licheniformis* J24 from Salt Lake, Tunisia, and *Bacillus safensis* from saline soils in Ganagavathi, India, both secrete potent chitinase and β-glucanase enzymes that effectively target *Fusarium* and *Sclerotinia* spp. [48,74]. Halophilic/halotolerant bacteria producing these enzyme combinations represent promising BCA, offering sustainable alternatives to chemical fungicides [256–258].

Multiple studies confirm that both β-glucanase and chitinase play crucial roles in antifungal defense, but their individual contributions and kinetics are often under-characterized. Evidence shows that the co-production or co-expression of these enzymes results in significantly stronger and broader antifungal activity than either enzyme alone, with synergistic effects observed in vitro and in planta across various crops. For example, transgenic wheat and tomato plants expressing both enzymes exhibited enhanced resistance to *Fusarium* and other pathogens, while combinations of purified enzymes from pea and cucumber inhibited a wider range of fungi than single enzymes, often through lysis of fungal hyphal tips [259].

Some studies have quantified enzyme kinetics, such as the characterization of chitinase and β-1,3-glucanase from *Trichoderma harzianum*, which demonstrated additive inhibition of *Sclerotium rolfsii* and provided specific kinetic parameters (Km and Kcat) for each enzyme. However, most research still lacks detailed kinetic analysis or direct linkage of enzyme activity levels to specific antifungal outcomes, making it difficult to determine the dominant enzyme in pathogen suppression for a given system [260]. Overall, the literature strongly supports co-expression strategies or the use of microbial consortia producing both enzymes for more effective and durable pathogen control in agricultural settings [259,261].

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2.7.5. Lipases

Lipases are a group of serine hydrolases that catalyze the hydrolysis of ester bonds between glycerol and long-chain fatty acids in lipids, generating free fatty acids, monoacylglycerols, diacylglycerols, or glycerol [262]. Belonging to the α/β-hydrolase fold superfamily, lipases generally exhibit specificity for acyl esters containing more than ten carbon atoms [263].

Beyond simple hydrolysis, these enzymes also catalyze transesterification, alcoholysis, and aminolysis reactions, typically achieving maximum activity at oil–water interfaces [264]. Microbial lipases, particularly those produced by bacteria, have drawn increasing interest due to their biochemical versatility, environmental resilience, and emerging roles in antifungal biocontrol. Depending on the producing organism and culture conditions, bacterial lipases can be secreted extracellularly, associated with membranes, or retained intracellularly [265]. They are widely distributed among bacteria, fungi, and yeasts [266], but bacterial lipases stand out for their stability, broad substrate specificity, and amenability to genetic or protein engineering, making them valuable tools for both industrial and agricultural applications [267,268].

In plant–pathogen systems, lipases contribute to host defense by degrading fungal membranes and triggering lipid-based immune signaling pathways [269]. Species of *Bacillus* including *B. subtilis*, *B. cereus*, and *B. pumilus* produce lipases capable of disrupting the membranes of fungal pathogens such as *Fusarium* and *Botrytis*. These enzymes target membrane phospholipids, leading to leakage of cellular contents and irreversible structural damage—one of the primary mechanisms underlying their antifungal action [212,270,271]. In addition, *Bacillus* species often co-secrete proteases, cellulases, and LPs that act synergistically with lipases to degrade complex fungal cell wall and membrane components, further enhancing biocontrol efficiency [270].

Halophilic/halotolerant bacteria represent promising sources of salt-tolerant lipases with broad substrate specificity and high catalytic efficiency. These enzymes maintain activity under elevated salinity, conferring a distinct advantage in salt-affected or degraded agricultural soils [265]. Functionally, bacterial lipases hydrolyze lipid membranes compromising fungal cell integrity and viability while remaining active across wide ranges of pH, temperature, and salinity. Many demonstrate dual activity on short- and long-chain lipid substrates, underscoring their enzymatic adaptability [212,272,273].

Bacillus strains from hypersaline environments such as mangroves, solar salterns, and salt ponds are particularly recognized for combining high lipase activity with strong antifungal potential [266]. For example, *Bacillus* strain QSLA1 from Egypt's Qarun Lake exhibited marked antagonism against *Fusarium oxysporum* f. sp. *lycopersici* while producing high levels of extracellular lipase, suggesting its potential for biological protection of tomato crops [54]. Similarly, *B. velezensis* FMH2 and *B. subtilis* subsp. *spizizenii* FMH45, isolated from the Sfax solar salterns in Tunisia, displayed both lipase and protease activity and effectively suppressed *Botrytis cinerea* infection in tomato fruits [73].

Comparable results have been reported for *B. subtilis* and *B. amyloliquefaciens* strains from marine and saltpan habitats, where high lipase production correlated with strong inhibition of *B. cinerea* and *Colletotrichum gloeosporioides* [57,144].

Despite these promising findings, research on lipase-mediated antagonism remains comparatively limited relative to other hydrolytic enzymes such as proteases or chitinases. Most available studies rely on qualitative assessments, such as halo formation or inhibition zone assays, rather than advanced lipidomic or membrane integrity analyses, thereby constraining mechanistic understanding.

Recent work has demonstrated that lipases derived from *Bacillus subtilis* can exhibit potent antimicrobial and antibiofilm activities against pathogens like *Staphylococcus aureus*,

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including disruption of the biofilm matrix and attenuation of virulence, suggesting wider biocontrol potential [274]. Furthermore, bacterial lipases have been shown to modulate host immune responses; for instance, secreted lipases from *S. aureus* inactivate immune-activating lipid ligands, helping the bacterium evade innate defenses underscoring the multifunctional nature of these enzymes in both hydrolysis and immunomodulation [275].

Comparative evidence suggests that halophilic/halotolerant *Bacillus* strains generally display broader substrate specificity and higher stability under saline stress than strictly halophilic genera, supporting their suitability for use in saline field conditions [274]. However, there is a lack of studies directly addressing the dual hydrolytic and signaling functions of lipases, such as their potential to stimulate jasmonate-related plant defenses under salt stress. Overall, while promising, the field would benefit from more quantitative and mechanistic studies, including lipidomic profiling and membrane integrity assays, to fully realize the potential of lipase-based antagonism in plant protection and microbial ecology [274,275].

3. Synergistic Microbial Mechanisms Driving Biocontrol Efficacy in Saline Soils

Microbial traits often interact synergistically to enhance biocontrol efficacy in saline soils, providing complementary functions that improve pathogen suppression, microbial competitiveness, and plant stress tolerance. Antagonism against fungal pathogens is rarely achieved through a single mechanism. Halophilic/halotolerant *Bacillus* strains typically employ multi-trait strategies, combining VOCs, siderophores, hydrolytic enzymes, LPs, and antibiotics to suppress pathogens while promoting plant growth.

EPS stabilize microbial communities, protect cells from osmotic stress, and enable sustained delivery of antifungal compounds, whereas hydrolytic enzymes degrade pathogen cell walls, increasing their vulnerability. VOCs extend pathogen suppression beyond the rhizosphere and modulate plant stress and growth signaling. Salinity stress reshapes microbial metabolism, often enhancing EPS and osmoprotectant production, while soil properties and plant root exudates further influence microbial community composition and function, selecting for salt-tolerant beneficial microbes. Co-inoculation of stress-adapted microbes with multifunctional traits, such as plant growth-promoting bacteria and fungi, demonstrates improved plant growth, nutrient acquisition, and stress mitigation, highlighting the advantage of multi-trait microbial consortia over single-strain inoculants for sustainable biocontrol in saline environments [276–280].

Salinity stress alters microbial metabolism by enhancing traits like EPS production and osmoprotectant synthesis, which help microbes survive osmotic and ionic stress. Soil physicochemical factors such as organic carbon content, pH, and electrical conductivity, along with plant root exudates, shape microbial community composition and function, selectively enriching salt-tolerant and beneficial microbes. Co-inoculation of stress-adapted microbes, particularly PGPB and arbuscular mycorrhizal fungi (AMF), shows synergistic effects by improving plant nutrient uptake, ion homeostasis, and stress tolerance, leading to enhanced growth under saline conditions. These microbial consortia modulate plant hormonal balance, antioxidant activity, and osmolyte accumulation, mitigating salt-induced damage and promoting systemic resistance. Studies demonstrate that dual inoculation with PGPB and AMF more effectively alleviates salinity stress than single inoculants, supporting sustainable crop production in saline soils. Overall, multi-trait, stress-adapted microbial consortia offer promising, eco-friendly strategies for saline soil remediation and improved plant resilience [281–286].

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Biocontrol and plant-beneficial microbes function as integrated consortia, where VOCs, siderophores, enzymes, and antibiotics mutually reinforce one another, modulate iron and nutrient availability, and activate plant immune responses. Presenting these interactions in a structured format (Table 2) highlights their complementary effects.

Table 2. Synergistic microbial mechanisms contributing to biocontrol.

Mechanism	How It Works/Interaction	Effect	Reference
Siderophores (iron scavenging)	Chelate Fe^{3+}, starving pathogens and improving plant iron uptake; directly suppress fungi	Strong pathogen suppression; improved plant iron nutrition	[287,288]
	VOCs can induce siderophore production, tightening iron competition and amplifying inhibition	Reinforced pathogen suppression	[289]
CWDEs and antibiotics	*CWDEs* combined with antiiotics and siderophores	Multi-layered suppression of pathogens (*Botrytis*, *Fusarium*)	[290]
	VOCs from neighboring microbes up-regulate genes for antibiotics and siderophore systems	Amplified inhibitory effects	[291]
VOCs	Act at a distance to inhibit pathogens, modulate microbial communities, and trigger plant defenses (ISR/SAR)	Inhibition of pathogens; activation of plant systemic resistance	[292,293]
	VOC mixtures often show synergistic antibiosis, more inhibitory than single compounds	Enhanced pathogen suppression	
	VOCs can modulate partners (e.g., enhancing siderophore production, P & K solubilization, IAA synthesis)	Promotes plant root growth and nutrient acquisition	

Abbreviations: VOCs, volatile organic compounds; *CWDEs*, cell wall-degrading enzymes; ISR, induced systemic resistance; SAR, systemic acquired resistance; IAA, indole-3-acetic acid.

4. Harnessing Halophilic/Halotolerant *Bacillus* for Sustainable Agriculture

Halophilic/halotolerant *Bacillus* strains have emerged as promising PGPR and BCAs, particularly suited to saline and stress-prone environments where conventional PGPRs often lose efficacy. These extremophilic bacteria enhance plant development, improve nutrient assimilation, and bolster tolerance to both salinity and heat stress across a variety of crops frequently performing on par with, or even surpassing, traditional PGPRs in challenging soils [294–298].

Beyond their growth-promoting effects, halophilic/halotolerant *Bacillus* species exhibit strong biocontrol capacity by interfering with pathogen signaling (quorum quenching), suppressing disease outbreaks, and offering protection against a broad spectrum of phytopathogens [299].

The ecological and agronomic value of these microorganisms lies in their sustainability and resilience. Unlike many conventional rhizobacteria, halophilic/halotolerant *Bacillus* strains can effectively colonize and remain metabolically active in degraded, arid, or saline soils environments typically unfavorable to microbial persistence. Their ability to sustain metabolic activity under osmotic and oxidative stress ensures the continuous expression of beneficial traits, such as the secretion of antifungal metabolites, siderophores, and the induction of systemic resistance in host plants [50,295,297]. Their persistence and adaptability under osmotic and oxidative stress enable consistent delivery of beneficial

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traits, including antifungal metabolite production, siderophore secretion, and induction of systemic resistance, even in marginal environments.

Such robustness makes halophilic/halotolerant *Bacillus* invaluable allies in the pursuit of sustainable agriculture and global food security, especially in regions increasingly affected by drought, soil salinization, and climate change [295,296]. By decreasing dependency on synthetic agrochemicals and supporting crop productivity under abiotic stress, these bacteria promote a shift toward more resilient, eco-friendly farming systems. However, their field performance is often strain-specific, emphasizing the need for rigorous selection, compatibility testing, and optimized formulation strategies to maximize synergistic interactions while minimizing potential antagonistic effects among microbial consortia [298,300].

5. Functional Diversity of Halophilic/Halotolerant Non-*Bacillus* Genera in Antifungal Biocontrol

While *Bacillus* spp. remains the most extensively studied halophilic/halotolerant BCAs owing to their endospore formation, metabolic versatility, and reliable performance across diverse environments several non-*Bacillus* genera contribute important and complementary antifungal traits. As outlined in Table 3, genera such as *Halomonas*, *Paenibacillus*, *Stenotrophomonas*, *Serratia*, and *Virgibacillus* and related taxa exhibit specialized mechanisms including distinctive VOC profiles, EPS production, and niche-adapted hydrolytic enzymes. These organisms often display greater strain dependency and variable field persistence, supporting their strategic use as targeted inoculants or as components of multi-strain consortia rather than as standalone biocontrol solutions.

Beyond *Bacillus*, several halophilic/halotolerant genera exhibit comparable adaptive and antagonistic capacities under saline stress. *Halomonas* spp. frequently produce hydroxamate-type siderophores such as desferrioxamine that sustain iron acquisition under high salinity and suppress *Fusarium* and *Penicillium* spp. [301,302]. *Stenotrophomonas rhizophila*, isolated from saline soils in Mexico, produces siderophores and VOCs that synergistically inhibit *Colletotrichum gloeosporioides* in mango [147]. Similarly, *Pseudomonas* spp. integrates siderophore-mediated iron depletion with antifungal metabolites such as HCN and phenazines, resulting in effective suppression of *Rhizoctonia* and *Fusarium* diseases [74,303].

Antibiotic- and VOC-mediated biocontrol is also prominent among non-*Bacillus* halophiles. *Pseudomonas stutzeri* isolated from hypersaline environments produces pyocyanin and phenazine-like compounds that inhibit *Fusarium oxysporum* and *Alternaria alternata* [303]. Halophilic *Streptomyces* strains synthesize structurally diverse macrolides and aminoglycosides with broad-spectrum antifungal activity [192]. Similarly, *Halomonas* species produce antimicrobial metabolites effective under high salinity, while *Stenotrophomonas rhizophila* secretes thermostable antibiotics active against *Colletotrichum* and *Botrytis* spp. [147].

Likewise, *Serratia marcescens* BKACT produces VOCs such as 2,4-di-tert-butylphenol that suppress *Fusarium foetens* [146], while *Paenibacillus* spp. and *Peribacillus* spp. release VOC blends including 2-furanmethanol, phenylacetonitrile, acetoin, and 2,3-butanediol that inhibit *Colletotrichum* and other phytopathogens [51,148]. *Brevibacterium halotolerans* JZ7 further contributes to VOC-mediated suppression of *F. oxysporum* in Chinese jujube through the production of 2,3-butanediol and fenretinide [149].

EPS-mediated stress protection represents another important non-*Bacillus* trait. Members of *Halomonas*, *Halobacillus*, and *Pseudomonas* produce abundant EPS that enhance rhizosphere colonization, soil aggregation, and plant tolerance to salinity while limiting pathogen invasion [304]. Notably, *Halomonas* sp. Exo1 from mangrove ecosys-

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tems improved rice salinity tolerance and inhibited *F. oxysporum* infection [302], while *Pseudomonas aeruginosa* PF23 suppressed *Macrophomina phaseolina* in salt-stressed sunflower through EPS production and biofilm formation [305]. However, comparative field-level data between EPS-producing non-*Bacillus* strains and *Bacillus*-based formulations remain limited.

Hydrolytic enzyme production is also widespread beyond *Bacillus*. Halophilic/halotolerant taxa from sabkhas, saline lakes, and hypersaline soils including *Halomonas*, *Virgibacillus*, *Oceanobacillus*, *Ochrobactrum*, *Paenibacillus* and *Serratia* produce salt-tolerant glucanases, proteases, lipases, and chitinases that retain antifungal activity against *Botrytis*, *Fusarium*, *Aspergillus*, and *Penicillium* spp. [32,160,211,254,306].

Collectively, these findings demonstrate that halophilic adaptation enhances antifungal functionality across diverse bacterial lineages, extending well beyond the traditional *Bacillus* model and supporting the strategic use of multi-genus consortia for biocontrol in saline agroecosystems.

Table 3. Representative halophilic/halotolerant non-*Bacillus* genera producing antifungal metabolites for biocontrol of phytopathogenic fungi.

Antifungal Metabolites	Biocontrol Bacteria	Geographical Origin	Target Fungal Pathogens	Disease/Crop	Reference
EPS	*Halomonas* sp. Exo1	Mangrove (*Avicennia marina*), Indian Sundarbans	*Fusarium oxysporum*	Rice	[302]
Siderophores; hydrolytic enzymes (β-1,3-glucanase, chitinase); VOCs (β-phenylethanol, dodecanal)	*Stenotrophomonas rhizophila*	CIBNOR Phytopathology Laboratory, Mexico	*Colletotrichum gloeosporioides*	Mango anthracnose	[147]
VOCs (2-furanmethanol, phenylacetonitrile, 2,4-dimethylpentanol)	*Paenibacillus* spp.	Colombian reefs	*C. gloeosporioides*	Anthracnose/Yam	[148]
VOCs (acetoin, acetic acid, 2,3-butanediol, isopentanol dimethyl disulfide, isopentyl and isobutanoate)	*Peribacillus* sp.	Saline and hypersaline environments	Multiple fungal spp.	Vine, potato, peach	[51]
VOCs (2,3-butanediol, fenretinide)	*Brevibacterium halotolerans* JZ7	Not reported	*F. oxysporum*	Chinese Jujube	[149]
Hydrolytic enzymes	*Paenibacillus* sp. PNM200	Colombian reefs	*F. oxysporum*	Tomato	[200]
VOCs (2,4-di-tert-butylphenol); hydrogen cyanide (HCN)	*Serratia marcescens* BKACT	Marine environment	*F. foetens*	Wheat	[146]
Hydrolytic enzymes	Halophilic bacteria (unidentified)	Great Sabkha and Chott, Northwestern Algeria	*Fusarium* spp.; *Penicillium* spp.	Not indicated	[307]
Hydrolytic enzymes	Halotolerant bacterium QSLA1	Qarun Lake, Egypt	*F. oxysporum* f. sp. *lycopersici*	Tomato	[54]
β-1,3–1,4-glucanase	*Halomonas meridiana* ES021	Saline ponds, Qarun Lake, Egypt	*Penicillium* sp.; *Aspergillus niger*	Not reported	[254]
CLPs (locillomycin A–C); bacillibactin	*Virgibacillus massiliensis*	Sebkha El-Meleh, Tunisia	*Fusarium* sp.	Not reported	[131]
HCN; hydrolytic enzymes (cellulase, amylase, lipase, proteases, urease, chitinase); VOCs (polyphenols)	*Serratia* spp. (SB6, CH11); *Halomonas* sp. (SB39)	Saline soils (Sabkha), Eastern Algeria	*Botrytis cinerea*; *Aspergillus niger*	Apple	[160]

Abbreviations: EPS, exopolysaccharides; VOCs, volatile organic compounds; HCN, hydrogen cyanide; CLPs, cyclic lipopeptides.

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6. Halophilic/Halotolerant *Bacillus*-Based BCAs Versus Conventional Fungicides

Comparative studies of microbial BCAs, including *Bacillus* spp., and synthetic fungicides reveal distinct trade-offs in efficacy consistency, environmental impact, resistance risk, and long-term sustainability.

6.1. Efficacy and Stability

Synthetic fungicides generally provide higher and more consistent disease control across a wide range of environmental conditions, whereas the performance of *Bacillus*-based BCAs is often more variable and strongly influenced by abiotic factors such as temperature, moisture, and formulation [308,309]. Seasonal fluctuations in efficacy are therefore common, and control levels may be lower than those achieved by top-performing fungicides [309]. Nonetheless, the ability of *Bacillus* species to form endospores confers advantages in formulation and storage stability relative to many other microbial BCAs, although overall shelf life remains inferior to that of synthetic fungicides [82,310].

6.2. Environmental Impact and Non-Target Effects

Conventional fungicides are associated with environmental risks, including soil and water contamination, non-target toxicity, and pesticide residues in agricultural products [82,311]. In contrast, microbial BCAs are generally regarded as environmentally compatible, exhibiting low toxicity, limited persistence, and reduced impacts on beneficial microorganisms and ecosystem functions. These attributes have contributed to increasing regulatory and agronomic interest in BCA-based and integrated disease management approaches [312–314].

6.3. Resistance Development

Resistance to single-site fungicides is widespread among plant-pathogenic fungi and represents a major constraint on chemical disease control [82,311,312]. *Bacillus*-based BCAs operate through multiple mechanisms, including antibiosis, nutrient competition, volatile organic compound production, and induction of plant defenses, which collectively reduce the likelihood of resistance development. Accordingly, BCAs are increasingly considered useful components of resistance management strategies when applied in rotation or in combination with reduced fungicide inputs [82,311,314].

6.4. Long-Term Sustainability

The long-term reliance on synthetic fungicides is increasingly limited by regulatory restrictions, resistance development, and environmental considerations [82,313,314]. *Bacillus*-based BCAs, particularly halophilic/halotolerant strains adapted to saline and stress-prone environments, are more compatible with sustainable and climate-resilient crop protection strategies. However, broader implementation will depend on improvements in formulation, field consistency, and integration into integrated pest management (IPM) programs [308,310].

7. In Vitro Antifungal Activity Versus in Planta and Field Performance of Halophilic/Halotolerant *Bacillus* Strains Under Saline Conditions

While halophilic/halotolerant *Bacillus* strains frequently exhibit strong antifungal activity in vitro, the literature consistently indicates that their performance under saline field conditions is lower and more variable; this in vitro field discrepancy is illustrated by the comparative examples summarized in Table 4, underscoring the need to interpret plate-

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based assays as an optimistic upper bound of biocontrol potential rather than a reliable predictor of field efficacy.

Table 4. In vitro antifungal activity versus in planta/field efficacy of halophilic/halotolerant *Bacillus* strains.

***Bacillus* Strain**	**Crop/Environmental Context**	**In Vitro Antifungal Activity (Main Assay vs. *Fusarium* spp.)**	**In Planta/Field Efficacy Under Saline Conditions**	**References**
Bacillus velezensis AF12; *Bacillus halotolerans* AF23	Tomato, saline soil	Strong antagonism against *F. oxysporum*; retention of PGP traits under 100–200 mM NaCl	Significant reduction in *Fusarium* wilt and increased biomass and chlorophyll content under 100–200 mM NaCl; partial but not complete disease suppression	[52]
Halotolerant *Bacillus velezensis* AP-3	Tomato, saline conditions	Strong dual-culture inhibition of *F. oxysporum* at 100 mM NaCl	~50% reduction in *Fusarium* wilt severity and improved plant growth under saline pot/greenhouse conditions	[315]
Bacillus halotolerans RFP57	Tomato and pea rhizosphere	Dual-culture inhibition of 44–61% vs. *F. oxysporum* f. sp. *lycopersici*; VOCs ≈ 55%; culture filtrates ≈ 33%	Only PGP traits and salt tolerance validated; greenhouse or field disease suppression not yet evaluated	[27]
Thermo-halotolerant *Bacillus cabrialesii* HB7	Tomato, saline conditions	Strong in vitro inhibition of multiple fungi, including *Botrytis cinerea*	Marked reduction in gray mold on post-harvest tomato fruits; improved germination and seedling growth under salinity	[202]
Bacillus licheniformis/*Bacillus paralicheniformis* TRQ65	Wheat, saline field	Salt-tolerant strain isolated from soils up to 6.4 dS m^{-1} EC; antifungal activity not primary selection criterion	Restored wheat growth in saline soil (6.4 dS m^{-1}) to near non-saline levels; disease suppression not quantified	[316]
Diverse halotolerant *Bacillus* spp.	Saline soils	Eight isolates showing antibiosis against *Sclerotium oryzae* and *Rhizoctonia solani*; activity linked to siderophores and enzymes	No in planta or field disease data; proposed as candidates for further validation	[317]

These observations are consistent with broader reviews highlighting that environmental filters such as soil pH, electrical conductivity, organic matter content, temperature, and interactions with native microbial communities often prevent laboratory-derived MIC or IC_{50} values from translating into proportional disease suppression in situ [316,318].

In saline soils in particular, osmotic stress, elevated ionic strength, and nutrient limitation can restrict root colonization, biofilm formation, and the production or persistence of antifungal metabolites by introduced *Bacillus* strains [14,295,318]. In addition, soil physicochemical properties and plant root exudates actively restructure rhizosphere microbial networks, frequently limiting the establishment and long-term activity of inoculated BCAs [316,318].

8. Discussion

Saline and hypersaline ecosystems, though rich in microbial diversity, remain among the least explored environments, particularly regarding their microbiomes and functional capacities. The integration of culture-dependent and -independent approaches has begun to reveal the complexity of these microbial communities, uncovering a wealth of halophilic/halotolerant bacteria with remarkable biotechnological potential [319].

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Advances in next-generation sequencing and bioinformatics have significantly improved our ability to decipher the intricate plant–microbe interactions occurring under extreme environmental conditions, including salinity and drought [320]. Such insights are increasingly important for the development of microbial strategies aimed at enhancing plant resilience to abiotic stress [321,322].

In recent decades, increasing attention has been directed toward the isolation and characterization of saline-adapted *Bacillus* strains with biocontrol activity against *Fusarium* spp. [323]. Their dual ability to suppress fungal phytopathogens while enhancing plant tolerance to salinity aligns well with the broader objectives of sustainable and environmentally friendly agriculture [324]. At a time when global food systems face mounting pressures from climate change, soil degradation, and population growth, such biological solutions are urgently needed. Sustainable disease management must now address both abiotic stress mitigation and pathogen suppression to secure crop productivity [325].

Importantly, salinity itself exerts a complex and dual influence on microbial biocontrol systems. Moderate salinity can act as a selective pressure that enriches halotolerant and metabolically versatile bacteria, particularly *Bacillus* species, which often display enhanced osmotic tolerance, improved biofilm formation, and activation of secondary metabolite pathways involved in pathogen suppression and plant stress alleviation. Under these conditions, salinity may indirectly enhance biocontrol efficacy by favoring stress-adapted microbial traits [326–329].

In contrast, excessive salinity imposes severe osmotic and ionic stress that can compromise bacterial survival, reduce rhizosphere colonization, and suppress the biosynthesis of key antifungal compounds such as LPs and volatile organic compounds. Elevated salt concentrations may further disrupt quorum sensing, cellular energy allocation, and plant–microbe signaling processes, ultimately diminishing biocontrol performance. At the community level, increasing salinity is generally associated with declines in soil microbial diversity and functional activity, with bacteria often being more sensitive than fungi, which, although comparatively more salt-tolerant, also exhibit reduced metabolic activity under extreme salinity. These contrasting effects underscore the need to define functional salinity thresholds and to tailor strain selection, formulation, and application strategies to maintain microbial activity and efficacy across saline environments [326–329].

This review highlights the vast bioprospecting potential of saline ecosystems as reservoirs of novel microbial taxa and bioactive metabolites with antifungal properties. The combined use of molecular biology, genomics, and microbial ecology has revealed that extremophiles from these habitats produce a diverse arsenal of metabolites such as LPs, VOCs, and hydrolytic enzymes that effectively inhibit a wide range of fungal pathogens. The growing body of research reflects a clear shift toward exploiting extremophile-derived BCA as central components of sustainable plant disease management strategies.

Over the past six years, numerous studies have consistently demonstrated the efficacy of halophilic and halotolerant bacteria in managing soil-borne fungal diseases under adverse environmental conditions. Their intrinsic ability to produce antifungal metabolites and stress-resilience factors makes them particularly attractive candidates for sustainable agricultural applications. Beyond crop protection, these microorganisms also hold significant promise across the green (agricultural), gray (environmental), red (pharmaceutical), and white (industrial) biotechnology sectors.

Halophilic/halotolerant bacteria thus represent valuable allies in developing climate-resilient agricultural systems. Their ability to thrive under saline stress while simultaneously promoting plant growth and suppressing pathogens offers practical, nature-based solutions to enhance productivity in degraded and salt-affected soils. Nevertheless, the transition from laboratory-scale research to large-scale field application remains a major

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challenge, requiring strong coordination among researchers, regulatory agencies, and agricultural practitioners.

Future progress depends on a well-defined commercialization roadmap, beginning with the omics-guided selection and optimization of highly potent bacterial strains. Integrated genomic, transcriptomic, and metabolomic analyses can identify elite candidates exhibiting superior antifungal activity, stress tolerance, and rhizosphere competence [202,330]. Linking these molecular traits with functional outcomes will accelerate the rational design of bioinoculants specifically adapted to saline and arid agroecosystems.

Once promising strains are selected, formulation development becomes critical to maintain microbial viability, stability, and efficacy under field conditions. The choice of carrier materials whether organic, polymeric, or mineral-based is key to ensuring shelf-life and functionality during storage and transport [331,332]. When developing microbial consortia, compatibility testing must verify synergistic interactions that enhance bioefficacy while avoiding antagonistic effects that could compromise performance [13,331–333].

Pilot-scale production and field validation constitute the next essential steps, involving multi-location trials to assess pathogen suppression, plant growth response, and inoculant persistence under real environmental conditions. These studies are fundamental for evaluating long-term efficacy, ecological safety, and yield stability [330–335]. Multi-year, multi-location trials are particularly recommended to assess durability and environmental impact [13,334]. Following validation, regulatory approval and biosafety assessments ensure that halophilic/halotolerant inoculants comply with national and international standards, posing no risk to native microbiota, non-target organisms, or soil health [331,332,334].

Ultimately, the successful adoption of these microbial technologies depends on their effective integration into industrial and agricultural systems. This requires close collaboration among academia, industry, and policymakers to facilitate technology transfer, streamline production, and foster farmer confidence [331,334]. Demonstration programs and outreach initiatives play a vital role in promoting awareness and acceptance of microbial bioformulations among growers and agricultural cooperatives [334].

Together, these steps define a pragmatic and scientifically grounded roadmap for translating halophilic/halotolerant bacteria from laboratory discovery to field application. Collectively, these findings support the view that in vitro assays are most valuable for early-stage screening and mechanistic insight, whereas reliable deployment of saline-adapted BCAs requires targeted formulation strategies, multi-location field trials, and deliberate selection for traits associated with robust root colonization, stress tolerance, and persistence in complex soil environments [318,336].

By coupling molecular innovation with ecological validation and stakeholder engagement, these extremophilic microbes hold substantial potential to advance modern biocontrol and plant growth-promotion strategies, paving the way for a new generation of sustainable, salt-resilient biofertilizers and biopesticides.

9. Challenges and Future Prospects

Although halophilic/halotolerant bacteria are increasingly recognized as valuable resources in agrobiotechnology, several scientific, technical, and logistical constraints still hinder their large-scale deployment. Unlocking their full potential and integrating them effectively into sustainable crop production systems will require a combination of fundamental research, applied innovation, and interdisciplinary collaboration. The following areas represent key priorities for future work:

I. Formulation and delivery constraints

Formulation and delivery remain the most immediate bottlenecks for field application. Many antifungal traits, including LPs and VOCs, exhibit reduced stability under fluctuating

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salinity, moisture, and temperature regimes typical of saline soils. Optimizing fermentation parameters, carrier materials, and encapsulation or controlled-release strategies is essential to ensure microbial persistence, sustained metabolite production, adequate shelf life, and compatibility with standard agricultural practices.

II. Bridging laboratory and field research

The limited availability of multi-location and long-term field trials is a major barrier to translation. Most studies remain confined to laboratory or greenhouse conditions that fail to capture the complexity and variability of saline agroecosystems. Field-based evaluations are urgently needed to assess biocontrol efficacy, rhizosphere persistence, non-target effects, and performance consistency across diverse soil types and climatic zones, particularly under climate-driven stress combinations.

III. Environmental and soil heterogeneity

Environmental variability strongly influences biocontrol reliability. Soil physicochemical properties such as electrical conductivity, pH, ionic composition, texture, and organic matter content affect microbial survival, rhizosphere colonization, and the stability and activity of antifungal metabolites. Spatial and temporal heterogeneity in saline soils further complicates predictability. Future research should explicitly incorporate these variables into experimental designs to improve robustness, reproducibility, and site-specific optimization.

IV. Mechanistic validation under field-relevant conditions

Most existing studies remain descriptive and are conducted under controlled conditions. Mechanistic investigations performed under realistic salinity gradients, soil chemistries, microbial competition, and climate-associated stresses are required to elucidate the regulation, expression, and durability of biocontrol traits. Such insights will enable the rational design of resilient bioformulations and optimized application strategies.

V. Integration of omics and systems biology

Advances in genomics, transcriptomics, proteomics, and metabolomics are essential for identifying salinity- and climate-responsive pathways underlying stress tolerance, antagonism, and plant growth promotion. Integrating multi-omics datasets through systems biology approaches can guide the selection, optimization, and potential engineering of elite, multi-trait strains with improved field resilience.

VI. Discovery and characterization of novel strains

Despite recent progress, the diversity of halophilic/halotolerant bacteria with biocontrol potential remains underexplored. Targeted exploration of saline and hypersaline environments, coupled with systematic screening across crops and pathogens, may yield strains with complementary antagonistic and plant growth–promoting traits. Comprehensive physiological, ecological, and genomic characterization is necessary to assess adaptability, functional stability, and biosafety.

VII. Genome editing and regulatory considerations

Genome editing tools, particularly clustered regularly interspaced short palindromic repeats (CRISPR–Cas) systems, offer opportunities to enhance stress tolerance, colonization efficiency, and antifungal activity. However, ecological risks, regulatory constraints, and public acceptance must be carefully evaluated prior to deployment. Responsible innovation will require transparent risk assessment and alignment with evolving biosafety and regulatory frameworks.

VIII. Expanding research and optimization efforts

Compared with conventional PGPR, research on halophilic/halotolerant microorganisms remains limited. Greater emphasis is needed on optimizing cultivation, fermentation, formulation, and delivery systems to improve microbial viability, metabolite production, and functional consistency under saline and climate-stressed conditions. Particular atten-

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tion should be paid to formulation stability, shelf life, and compatibility with irrigation regimes, fertilizers, and pesticides commonly used in salt-affected agroecosystems.

IX. Climate change as a central driver for saline-adapted BCAs

As climate change continues to intensify soil salinization and compound abiotic stresses, the limitations of conventional chemical fungicides and non–stress-adapted microbial inoculants are expected to become more pronounced. In this context, halophilic/halotolerant BCAs particularly *Bacillus* species represent climate-resilient alternatives capable of sustaining antagonistic activity and plant growth–promoting functions under combined salinity, drought, and heat stress. Their integration into climate-smart and sustainable crop protection strategies is therefore likely to play an increasingly important role in future agricultural systems.

10. Conclusions

The increasing prevalence of soil salinization, climate variability, and fungal plant diseases poses a serious challenge to global food security, necessitating the development of sustainable and resilient crop protection strategies. This review highlights the growing potential of halophilic/halotolerant bacteria, particularly *Bacillus* species, as effective biological control agents against *Fusarium* and other major phytopathogenic fungi. Their ecological adaptability, stress tolerance, and capacity to produce diverse antifungal metabolites position them as valuable alternatives to chemical fungicides, especially in saline and degraded soils where conventional approaches often fail.

Halophilic/halotolerant *Bacillus* spp. exert biocontrol through a coordinated suite of complementary mechanisms, including the production of antifungal LPs and VOCs, siderophore-mediated iron competition, secretion of hydrolytic enzymes, nutrient competition, and the induction of plant systemic resistance. The integration of these traits enables effective pathogen suppression while simultaneously enhancing plant growth and tolerance to salinity and other abiotic stresses.

Among the most promising candidates are *Bacillus* salt-adapted strains, which have demonstrated both BCAs and PGPR effects under saline conditions, highlighting saline and hypersaline environments as underexplored reservoirs of resilient BCAs.

Despite these advantages, large-scale agricultural deployment remains constrained by strain-specific performance, formulation challenges, and limited multi-location field validation. Addressing these limitations will require targeted strain selection based on rhizosphere competence and stress tolerance, advances in formulation and delivery technologies to enhance field consistency, and rigorous, multi-environment field trials to assess efficacy across diverse agroecosystems. Equally important is strengthened interdisciplinary collaboration among microbiologists, formulation scientists, agronomists, industry stakeholders, extension services, and regulatory agencies to accelerate technology transfer, product development, and adoption.

Such coordinated efforts are essential for translating laboratory discoveries into scalable, economically viable microbial biocontrol solutions and for advancing climate-resilient, environmentally sustainable agriculture in salt-affected systems.

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Abbreviations

The following abbreviations are used in this manuscript:

2,4-DBTP	2,4-dibromothiophenol
ACC deaminase	1-aminocyclopropane-1-carboxylate deaminase
AMF	Arbuscular mycorrhizal fungi
ARGs	Antibiotic resistance genes
BCAs	Biological control agents
CRISPR	Clustered regularly interspaced short palindromic repeats
CWDEs	Cell wall-degrading enzymes
EC	Enzyme Commission
EPS	Exopolysaccharides
GC–MS	Gas chromatography–mass spectrometry
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
IC_{50}	Half maximal inhibitory concentration
IPM	Integrated pest management
ISR	Induced systemic resistance
kDa	Kilodalton
LC–MS	Liquid chromatography–mass spectrometry
LPs	Lipopeptides
MIC	Minimum inhibitory concentration
MVOCs	Microbial volatile organic compounds
PGPB	Plant growth-promoting bacteria
PGPR	Plant growth-promoting rhizobacteria
ROS	Reactive oxygen species
SPME	Solid-phase microextraction
VOCs	Volatile organic compounds

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Biotechnological Potential of Native Thermophilic Microorganisms for Creating a Soil Biomeliorant from Poultry Manure

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Abstract

The aim of the study was to evaluate the biotechnological potential of thermophilic microorganisms isolated from chernozem soil during composting of poultry manure. The efficiency of the strains was determined by their effect on organic matter degradation, humification intensity, and nitrogen accumulation. The correlation between the quality indicators of the composting process was assessed with the gross values, taking into account the proportion of compost fractions. The strains were identified as: *Aeribacillus pallidus* KCTC 3564^T (cellulolytic), *Neobacillus sedimentimangrovi* FJAT-2464^T, *Aeribacillus composti* N.8^T, *Caldifermentibacillus hisashii* N-11^T (nitrogen fixers), *Acinetobacter pittii* CIP 70.29^T, and *Pseudomonas plecoglossicida* NBRC 103162^T (nitrifies). It was found that all the bacteria increase the proportion of small fractions by 19.0–19.9%. The gross content of humic acids increases under the influence of nitrifiers (15.5%) and nitrogen fixers (5.5%). The total nitrogen content increases under cellulolytics (13.8%) and nitrogen fixers (20.2%). The smallest fraction ($\leq$0.25 mm) in nitrogen fixers and nitrifying variants has the greatest bioreclamation properties, by 16.4% ($p < 0.001$) and 12.9% ($p < 0.001$). Targeted microbial strains provide the direction of the transformation processes during biocomposting. It can also be concluded that assessing the quality of composting based on the fraction distribution can be a promising element of the biofermentation process monitoring.

Keywords: thermophilic bacteria; nucleotide sequence of genes; 16S rRNA; biofermentation; poultry manure

1. Introduction

When discussing organic raw materials obtained in the livestock industry, definitions should reflect their status. Until recently, the term "livestock waste" was widely used in relation to manure, which, given its agrobiotechnological potential, was not objective. Poultry manure is a particularly valuable by-product of animal husbandry from an agronomic perspective. Approximately 20 million tons of poultry manure are generated annually in the Russian Federation [1]. The fertilizing potential of manure (natural moisture) in terms of mineral components with a nitrogen, phosphorus, and potassium content of 2.1%, 1.44%, and 0.64%, respectively [2] is comparable to 420 thousand tons of the active substance of mineral fertilizers for nitrogen, 288 thousand tons for phosphorus, and

128 thousand tons for potassium. Manure is also rich in mesoelements that are important for plant nutrition. Thus, according to [3], manure contains 0.67% magnesium oxide, 1.92% calcium, and 0.38% sulfur, which is equivalent to 134, 384, and 76 thousand tons of the active substance, respectively.

The value of poultry manure is not limited to its mineral nutrient content. As carbon-containing substances, they provide energy for both native soil microflora and those introduced with commercial biopreparations. Microorganisms are a significant factor in soil formation, which is essential for restoring the carbon sequestration capacity of the land surface, which has significantly decreased since 2023 and is possibly responsible for the continued rise in atmospheric temperature. According to [4], June 2023, 2024, and 2025 recorded the highest total temperatures in the history of meteorological observations.

The climate effect of organic fertilizers from poultry manure also includes reducing the carbon footprint of crop production by partially or completely replacing mineral fertilizers. The contribution of the mineral fertilizers to the overall carbon footprint of grain crops can reach 25% [5], and in the structure of the carbon footprint of the final product (wheat bread), it accounts for up to 15% [6].

The effectiveness of using poultry manure as fertilizer is largely determined by the processing technology [7]. The choice of technology must be consistent with both environmental and economic considerations. One of the most accessible technologies for processing poultry manure is solid-state aerobic fermentation (composting) [8,9]. The "noble manure" technologies [10] have significant potential for further modernization. Improvements of this technology are largely associated with the selection of specialized microbial strains to produce materials that meet sanitary and epidemiological requirements [11–15] and to improve their quality [16–18].

The use of consortia is aimed at achieving a controlled composting process, depending on the goals and characteristics of the raw material. For example, a bacterial consortium with an index of 5842, consisting of lactic acid bacteria and streptococci (*Streptococcus thermophilus*, *Streptococcus bovis*, *Lactobacillus salivarus* var. *salicinicus*, *Lactobacillus salivarus* var. *salivarius*, *Lactobacillus x* (*acidophilus*)), has been proposed for the safe processing of poultry manure [19]. To reduce unpleasant odors and accelerate the decomposition of poultry manure, it is recommended to apply lactic acid bacteria, photosynthetic bacteria, and yeast locally [20]. A consortium of microorganisms (*Anoxybacillus kamchatkensis* AS-GOS-2, *Bacillus amyloliquefaciens* PL-1, *Aneurinibacillus thermoaerophilus* PL-5, *Brevibacillus brevis* PM-3, and *Pseudoxanthomonas taiwanensis* PM-cell) isolated from animal waste and activated sludge has been created for the joint composting of organic waste from poultry farming and wood processing [10].

The concept of creating microbial consortia for biofermentation of poultry manure in order to create soil fertilizers should take into account the main stages of transformation of organic raw materials and the biophilic elements they contain. The most important stages in composting material are the destruction of primary organic matter and its humification; therefore, it is logical to select bioagents that accelerate these processes. Such bioagents can include specific strains of thermophilic microorganisms, the use of which meets economic interests by shortening the cycle time for creating the final fertilizer. During the humification process, nitrogenous compounds are actively transformed, and therefore, the risk of losing valuable plant nutrients in gaseous form can be reduced by selecting specific microorganisms involved in the nitrogen cycle. The concept should take into account quantitative indicators of the final product: the degree of product degradation (proportion of fine fraction (%)), the degree of humification of the material (proportion of humic substances, mg/kg), the content of total nitrogen (%) and its mineral forms (g/kg), as well as the bioreclamation properties of the final product.

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The aim of the study was to evaluate the biotechnological potential of thermophilic microorganisms of various physiological groups isolated from ordinary chernozem during the composting of poultry manure with aerobic solid-state fermentation.

2. Materials and Methods

Soil samples for the isolation of target strains were collected from ordinary chernozem in the steppe zone of the Kabardino-Balkarian Republic from a long-term observation site included in the Geographic Network of Long-Term Experiments (Station No. 037, 1948) for monitoring the effects of various types of fertilizers.

Thermophilic Cellulolytic Bacteria (CB^T) were isolated using the Koch plate method, followed by cultivation under different temperature conditions on Hutchinson-Clayton growth medium with the following composition: glucose—10 g/L; $NaNO_3$—2.5 g/L; K_2HPO_4—1.0 g/L; $MgSO_4 \times 7H_2O$—0.3 g/L; NaCl—0.1 g/L; and $CaCl_2 \times 4H_2O$—0.1 g/L; $FeCl_3 \times 6H_2O$—0.01 g/L.

Thermophilic nitrogen-fixing microorganisms were isolated on Ashby's selective medium with the addition of Fedorov's trace elements of the following composition: sucrose—20.0 g/L; K_2HPO_4—0.2 g/L; $MgSO_4 \times 7H_2O$—0.2 g/L; NaCl—0.2 g/L; K_2SO_4—0.1 g/L; $CaCO_3$—5.0 g/L; agar—2.0 g/L; 1 mL of stock solution containing H_3BO_3 in an amount of 5.0 g/L; $(NH_4)MoO_4 \times 2H_2O$—5.0 g/L; $ZnSO_4 \times 7H_2O$—0.2 g/L; KI and NaBr—0.5 g/L each; and $Al_2(SO_4)_3 \times 18H_2O$—0.3 g/L in 1000 mL of distilled water. The suspension was incubated for 24 h at 47 °C in a thermoshaker (160 rpm). Pure cultures were isolated by subsequent transfer to a solid nutrient medium—fish protein hydrolysate [21].

Nitrifying Bacteria (NB^T) used to limit nitrogen losses in gaseous form during fermentation of poultry manure, isolated under different temperature conditions on S.N. Vinogradsky's selective nutrient medium consisting of: $(NH_4)_2SO_4$—2.0 g/L; K_2HPO_4—1.0 g/L; $MgSO_4$—0.5 g/L; $FeSO_4$—0.4 g/L; NaCl—2.0 g/L; and tap water. Pure cultures were isolated by subsequent transfer to solid media [21]. The manufacturer of micronutrient components for nutrient media is "Khimreaktivsnab" JSC, Ufa, Russia. The manufacturer of organic nutrient media is "Agat-Med" LLC, Balashikha, Russia.

The thermophilic status of the bacteria was confirmed by transferring them to solid nutrient media (GMF-agar) and incubating them at a wide range of temperatures: 20–65 °C. It was found that four strains (NFB^T2, NFB^T9, NFB^T10, CB^T) show active growth at temperatures between 45 and 60 °C, with no growth below 40 °C. The optimal range is 47–50 °C. Nitrifying strains (NB^T1, NB^T2) were less resistant to elevated temperatures, with no growth above 50 °C, and the ability to grow at 20 °C. Nitrifying strains (NB^T1, NB^T2) were less resistant to increased temperatures, did not grow above 50 °C, and were capable of growing at 20 °C.

The strains were identified based on the nucleotide sequence of the 16 rRNA genes. For composting poultry manure with the isolated microorganisms, an experimental compost pile was placed on a concrete pad with the following dimensions: length—36 m, width—1.5 m, and height—1 m (Figure 1a). Microorganisms were added with a knapsack sprayer at a rate of 1 L of culture liquid ($2–4 \times 10^8$) per 1 ton of dry poultry manure. Given the bulk density of fresh poultry manure of 0.3 t/m^3, 4.5 m of stack was allocated for each treatment. Cellulose-degrading microorganisms were added as a monoculture. Three strains of nitrogen-fixing microorganisms were added together—0.35 L/t of each strain, and two strains of nitrifying microorganisms were added at 0.5 L/t of each one. Samples were taken from each segment and analyzed in three replicates. Manure pile turning was carried out using a STEVIMAN SX-61 (LLC «Small-Scale Mechanization», Moscow, Russia) snow blower (Figure 1b). The turning frequency was 110 h, in accordance with recommendations [22]. The pile moisture content was maintained at 60–65% and monitored

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using a professional MS-7828 (AZ Instrument Corp, Taichung, Taiwan) soil moisture meter. The ambient temperature during the experiment (June–July 2025) ranged from 29 to 38 °C during the day to 22–26 °C at night. The composting period lasted 21 days. An average biomass sample for analysis was collected by cutting across the entire height of the pile (Figure 1c). The composted material was located in the Kabardino-Balkarian Republic, Urvan District, Kakhun village, on the territory of the former collective farm "Kakhunsky".

Figure 1. View of the experimental pile (**a**), turning over the pile (**b**), and sampling for analysis by cutting along the entire height of the pile (**c**).

The rate of organic matter destruction was determined by sifting air-dried compost through sieves of ≤0.25 mm, 0.5 mm, 1.0 mm, and ≥2.0 mm. The compost was first physically destroyed in a Stegler LM-250 (Shenzhen Bestman Instrument CO., LTD, Shenzhen, China) laboratory mill for 20 s. The degree of mineralization of the organic matter in poultry manure under the influence of the soil microbial strains was determined by measuring the proportion of organic carbon in the fractions. Organic carbon was determined using the Tyurin method [23].

The degree of humification was determined by measuring the humic acid (HA) content spectrophotometrically in each fraction differentially [24]. The nitrogen status of the compost was determined based on the total nitrogen content and the proportion of mineral nitrogen compounds. The total nitrogen content was determined in accordance with [25] using an AKV-10 semiautomatic Kjeldahl apparatus using the titrimetric method. The nitrate content was determined using the method of V.B. Zamyatina [26,27]. The content of the ammonium form of nitrogen [28] was determined using the Nessler reagent [26].

The bioreclamation properties of the compost samples were determined fractionally. For this purpose, a laboratory experiment was conducted with 2.3 g of each fraction (≤0.25 mm, 0.5 mm, 1.0 mm, and ≥2.0 mm) added to trays containing 500 g of ordinary chernozem in 3-fold repetition. The change in the biological response of the soil to the application of each compost fraction after 14 days was assessed by the carbon content of microbial biomass and the invertase activity of the soil.

Statistical analysis was performed using Python 3.10 software with SciPy and Statsmodels libraries. Due to the small sample size (n = 3), the normality of the dis-

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tribution was checked using the Shapiro–Wilk test, where the normality criterion was $p > 0.05$. The homogeneity of the variances between the compared groups was assessed using the Levene test, where the assumption of homogeneity of variances was made at $p > 0.05$. When all the necessary conditions are met, a one-way analysis of variance (One-way ANOVA) was performed to assess the effect of various microorganisms (CBT, NBT, and NFBT) on all the studied indicators. When the conditions were violated, the non-parametric Kruskal–Wallis test was performed. The significance levels were as follows: $p < 0.001$ for highest significance, $p < 0.01$ for high significance, $p < 0.05$ for significance, and $p \geq 0.05$ for non-significance. Tukey's test (Tukey HSD) was used for multiple comparisons with the control. The significance level was set at $p = 0.05$. The data were presented as the mean ± standard deviation of three replicates. Graphical data that do not include standard errors reflect the result of recalculating the primary data based on the fraction data. Pearson correlation analysis was performed to study the relationship between the indicators of total nitrogen and humic acids, total nitrogen and mineral forms of nitrogen in the compost, where the results were classified according to the Chaddock scale. The statistical significance of the correlation coefficients was determined by comparing the critical value of Student's *t*-test for a significance level of $p = 0.05$ with the empirical value of Student's *t*-test.

3. Results and Discussions

3.1. Cultural and Physiological-Biochemical Properties of Isolated Microorganisms

All isolated microorganisms used in the study were Gram-positive bacteria belonging to five different genera (Table 1).

Table 1. Phylogenetic analysis of nucleotide sequences of 16S rRNA gene fragments of soil microorganism strains.

Strains	Closely Related Type Strain	No GenBank	Similarity, %	Number of Nucleotides, Units
CB^T	*Aeribacillus pallidus* KCTC 3564^T	CP017703	100	1272
NFB^T2	*Neobacillus sedimentimangrovi* FJAT-2464^T	MN963926	100	1384
NFB^T9	*Aeribacillus composti* $N.8^T$	LT594972	100	1293
NFB^T10	*Caldifermentibacillus hisashii* N-11^T	AB618491	100	814
NB^T1	*Acinetobacter pittii* CIP 70.29^T	APQP01000001	100	792
NB^T2	*Pseudomonas plecoglossicida* NBRC 103162^T	BBIV01000080	100	790

CB^T are spore-forming, solitary, round bacteria that are urease-, phosphatase-, and amylase-positive, catalase-negative, not proteolytically active, and utilize nitrogen from nitrate salts and are capable of degrading cellulose. Their optimal growth temperature is 45–55 °C, with growth occurring at temperatures up to 65 °C and no growth at 30 °C.

NFB^T2 are spore-forming, rod-shaped bacteria that are catalase-, phosphatase-, urease-, and amylase-negative, possessing proteolytic activity, atmospheric nitrogen-fixers, and able to utilize ammonium salts. Their optimal growth temperature is 47–50 °C, with no growth at 30 °C.

NFB^T9 are spore-forming, solitary, elongated bacteria that are urease-negative, catalase-, phosphatase-, and amylase-positive, lacking proteolytic activity, and assimilate nitrogen from atmospheric air. Optimum growth temperature is 47–50 °C; no growth occurs at 30 °C.

NFB^T10 are spore-forming, solitary, round-ended bacteria that are urease-negative, catalase-, phosphatase-, and amylase-positive, lacking proteolytic activity, and assimilate

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nitrogen from atmospheric air. Optimum growth temperature is 47–50 °C; no growth occurs at 30 °C.

NB^T1 are thermotolerant, non-spore-forming, round, solitary bacteria that are urease- and catalase-positive, phosphatase- and amylase-negative, and possess proteolytic activity. They assimilate nitrogen from atmospheric air, nitrates, and ammonium salts, and possess the ability to mobilize phosphates. The optimal growth temperature is 30–40 °C, with a maximum temperature of up to 50 °C.

NB^T2 are thermotolerant, non-spore-forming, solitary, elongated bacteria that are urease-, catalase-, and phosphatase-positive, and amylase-positive, possessing proteolytic activity. They assimilate nitrogen from nitrates and ammonium salts and possess the ability to mobilize phosphates. The optimal growth temperature is 30–40 °C, with a maximum temperature of up to 50 °C.

3.2. Degree of Organic Matter Destruction

Accelerating the decomposition of organic matter is a fundamental element of composting technologies. However, the authors did not find any use of the proportion of individual fractions (fragmentation) in assessing the composting process of poultry manure in the scientific literature. It is logical to assume that an increase in the proportion of the smallest fractions reflects the intensity of biochemical reactions in the compost. The mechanism of accelerated fractionation of manure under the influence of the studied thermophilic microorganisms may be the same as that of thermophilic actinobacteria, which can use recombinant hydrolases to degrade even synthetic polymers [29–31]. At the same time, the authors propose separating the concepts of "decomposition," which reflects deeper processes of biochemical transformation of organic matter, such as humification, from "destruction," which reflects the quantitative expression of physical parameters such as particle size of the composted material. It can also be expected that the particles reduced by the enzymatic apparatus of microorganisms have a greater surface area in contact with the compost microflora, which participates in the further transformation of carbon and nitrogen-containing compounds.

The fractions presented in the table can be roughly divided into small ($\leq$0.25 mm and 0.5 mm), reflecting the intensity of decomposition, and large (1.0 mm and $\geq$2.0 mm), the proportion of which reflects the advisability of continuing the composting process. The analysis results demonstrate the influence of the studied microorganisms on the destruction of organic matter with varying degrees of intensity (Table 2).

One-factor analysis of variance revealed statistically significant differences between the experimental options for all the studied fractions. The most pronounced differences were observed for the 0.5 mm ($p < 0.001$) and $\geq$2.0 mm ($p < 0.001$) fractions, indicating the impact of the studied microorganisms on the degradation of poultry manure. For the $\leq$0.25 mm and 1.0 mm fractions, the differences were also statistically significant ($p < 0.05$), but less pronounced.

Considering the effect of the isolated strains on the destruction of organic matter in poultry manure, it should be noted that, based on the sum of fractions $\leq$ 0.25–0.5 mm, the strains of nitrifying thermotolerant microorganisms were the most effective, increasing the fragmentation rate of organic matter by 9.7%. A cellulose-degrading strain and a consortium of thermophilic nitrogen-fixing microorganisms increased this rate by 7.9% and 8.8%, respectively. It was expected that cellulolytic microorganisms would be more aggressive in increasing the proportion of degraded matter. The greater effectiveness of nitrifying microorganisms may be due to their ability to release mineral nitrogen, which acts as an activator of the native cellulolytic microflora of poultry manure. The proportion of undestroyed or slightly destroyed organic material (fraction $\geq$ 2.0 mm) was observed in the

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control variant (20.8%), which turned out to be 18.8–30.8% higher than under the influence of the studied microorganisms. The proportion of undisturbed or slightly decomposed organic material (fraction ≥ 2.0 mm) was noted in the control variant (20.8%), which turned out to be 18.8% ($p = 0.001$), 29.7% ($p < 0.001$), and 30.7% ($p < 0.001$) higher than under the action of nitrifying, nitrogen-fixing, and cellulosolitic microorganisms, respectively.

Table 2. Degree of decomposition of poultry manure after biocomposting.

Fractions, mm	Variants	Degree of Decomposition, %	SD, %	Variation in Relative Control, %	*p*-Value	Statistical Significance ($p < 0.05$)
≤0.25	Start	16.0	0.63	-*	-	-
≤0.25	Control	24.4	0.93	-	-	-
≤0.25	CB^T	22.8	1.03	−6.6	0.1136	No
≤0.25	NB^T	26.3	1.27	7.8	0.1068	No
≤0.25	NFB^T	24.4	0.93	0	1.0000	No
0.5	Start	16.3	0.70	-	-	-
0.5	Control	19.9	0.84	-	-	-
0.5	CB^T	25.0	0.99	25.6	0.0024	Yes
0.5	NB^T	22.3	1.04	12.1	0.0370	Yes
0.5	NFB^T	23.8	0.44	19.6	0.0021	Yes
1.0	Start	28.7	1.57	-	-	-
1.0	Control	34.9	1.24	-	-	-
1.0	CB^T	37.8	0.91	8.3	0.0307	Yes
1.0	NB^T	34.5	1.56	−1.2	0.7420	No
1.0	NFB^T	37.1	0.90	6.3	0.1700	No
≥2.0	Start	39.0	0.85	-	-	-
≥2.0	Control	20.8	0.59	-	-	-
≥2.0	CB^T	14.4	0.58	−30.7	0.0002	Yes
≥2.0	NB^T	16.9	0.33	−18.8	0.0006	Yes
≥2.0	NFB^T	14.6	0.33	−29.7	0.0001	Yes

* Comparisons are referenced to the Control value. The initial mean value (Start) serves as a baseline to demonstrate changes over time. The "-" symbol in the table indicates that no direct comparison was performed between the Start and Control conditions.

3.3. Organic Matter Mineralization by Fraction

During composting, active decomposition of organic matter occurs, and the degree of mineralization is estimated by the proportion of total carbon in the biomass. The analysis data (Figure 2a) demonstrate that the introduction of the studied strains into the composted material can accelerate the mineralization of organic matter. The results of the analysis of variance showed that statistically significant differences between the options were established in all fractions ($p < 0.001$).

The minimum degree of mineralization (43.2% on average) was observed in the control variant, in which the proportion of organic carbon increased by 33.4–51.9% as the fraction size increased. Under the influence of nitrifiers, the reduction in organic carbon in all fractions varies between 21.7% ($p < 0.001$) and 36.9% ($p < 0.001$), while the action of cellulolytic enzymes results in a degradation of 9.1% ($p = 0.028$) to 29.9% ($p < 0.001$). The content of organic carbon in the 0.5 mm, 1.0 mm, and ≥2.0 mm fractions decreases by 29.3% ($p < 0.001$), 23.3% ($p = 0.003$), and 45.3% ($p < 0.001$), respectively, under the influence of nitrogen fixators.

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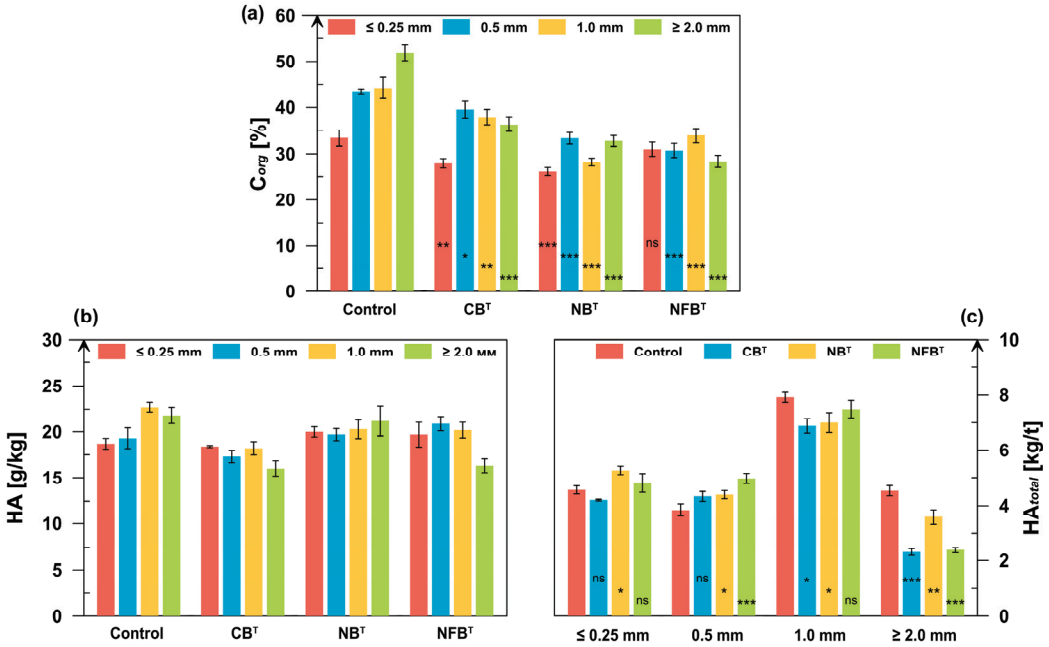

Figure 2. Content of (**a**) organic carbon by fractions, (**b**) humic acids, and (**c**) total of humic acids in different fractions of compost. Designations in the figures: C_{org}—organic carbon, %; HA—humic acids, g/kg; HA_{total}—total humic acid content, taking into account the fraction, kg/t; Control—control variant; CB^T—variant with cellulolytic bacteria; NB^T—variant with nitrifying bacteria; NFB^T—variant with nitrogen-fixing bacteria. The compost fractions studied were: ≤0.25 mm, 0.5 mm, 1.0 mm, and ≥2.0 mm. Levels of *p*-values: very high (***, $p < 0.001$), high (**, $p < 0.01$), significant (*, $p < 0.05$), not significant (ns, $p \geq 0.05$).

3.4. Organic Matter Humification by Fraction

The influence of microorganisms on humic substance accumulation varies by fraction (Figure 2b). The maximum amount of HA in the smallest fraction (≤0.25 mm) is formed under the influence of nitrifiers (20.0 g/kg) and nitrogen fixers (19.7 g/kg), which is 7.0% and 5.3% higher than in the control variant. In the 0.5 mm fraction, nitrogen fixers increase the HA content by 8.3%.

As fractions increase (1.0 and ≥2.0 mm), the maximum HA content is observed in the control variant—22.7 and 21.8 g/kg, respectively. The impact of cellulose-degrading microorganisms on the humification process is minimal.

At the same HA value in the ≤0.25 mm fraction as in the control, a decrease of 10.4%, 19.8%, and 26.6% was observed in the 0.5 mm, 1.0 mm, and ≥2.0 mm fractions, respectively. These data may serve as the basis for a hypothesis on revising the concept of using cellulolytics in the processing of animal byproducts. Thus, they can be added to the composted mass in combination with microbial strains of other physiological groups or used at later stages of fermentation. A more objective assessment of the humus status of fractions can be made based on their total HA content, calculated based on the HA concentration (g/kg) and the fraction's proportion (%) in the compost (Figure 2c). For greater reliability, statistical processing was carried out with data obtained after recalculation to the fraction share. This approach is also proposed for the indicators of total and mineral nitrogen.

The figure shows that the smallest fraction (≤0.25 mm) in 1 ton of compost accumulates between 4.2 and 5.26 kg of HA, depending on the microorganisms used. The maximum effect is achieved with the addition of nitrifying 5.26 kg/t microorganisms. This is 15.4% ($p = 0.01$) higher than in the control. In the next-largest fraction, 0.5 mm, the maximum accumulation of HA occurs under the influence of nitrogen-fixing microorganisms—4.97 kg/t, which is 29.4% ($p < 0.001$) higher than in the control (4.56 kg/t). The effects of

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nitrifiers were equal to 4.39 kg/t, which is 14.3% ($p = 0.03$), higher than the control value (3.84 kg/t).

As the fraction size increases, the total HA content in the experimental variants (CB^T and NB^T) decreases relative to the control value on 13.1% ($p = 0.01$) and 11.6% ($p = 0.02$). This trend persists in the ≥2.0 mm fraction. The maximum content (4.53 kg/t) was found in the control variant, which is 26.5% ($p = 0.001$) –96.7% ($p < 0.001$) higher than in the variants using microorganisms.

3.5. Total Nitrogen Content in Compost Fractions

The preservation of nitrogen in compost during the processing of waste by-products is an important quality indicator. The analysis results (Figure 3a) indicate variations in compost nitrogen content by fraction depending on the physiological characteristics of the microbial strains used in biofermentation. In the ≤0.25 mm fraction, the nitrogen content was higher than in the control by 13.8% (CB^T), 4.8% (NB^T), and 20.2% (NFB^T). In the 0.5 mm and 1.0 mm fractions, the difference between treatments narrowed. The maximum total nitrogen content (1.98%) relative to the control (1.77%) was observed with the use of nitrogen fixers in the 0.5 mm fraction, with a relative difference of 11.9%. In the 1.0 mm fraction, the difference was 10.7%.

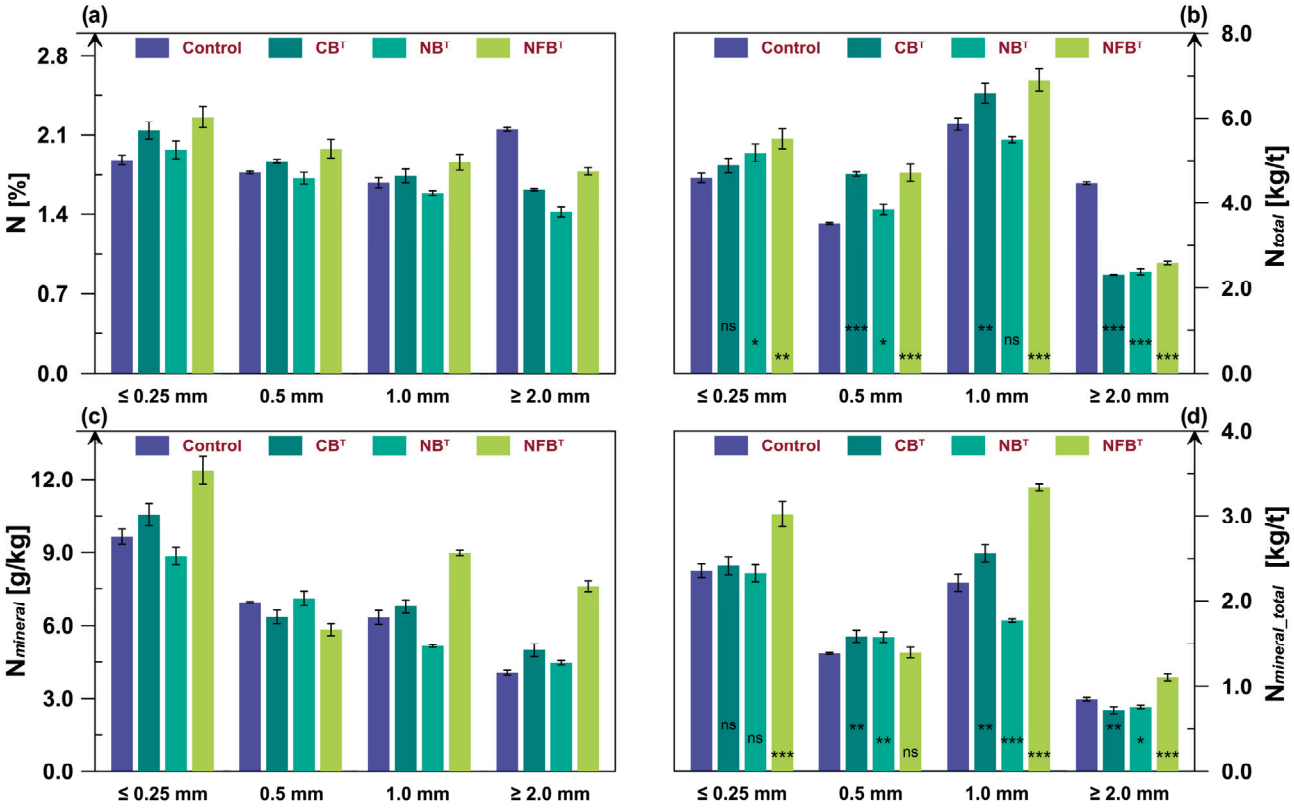

Figure 3. Relative content of (**a**) total nitrogen by fractions, (**b**) total content of total nitrogen in compost, (**c**) mineral forms of nitrogen by fractions, and (**d**) total content of mineral forms of nitrogen, taking into account the proportion of fraction. Designations in the figures: N—nitrogen in compost fractions, %; N_{total}—total nitrogen content, taking into account the fraction of fractions in kg/t; $N_{mineral}$—mineral forms of nitrogen ($NO_3 + NH_4$), g/kg; $N_{mineral_total}$—total content of mineral forms of nitrogen ($NO_3 + NH_4$), taking into account the proportion fractions, kg/t; Control—control variant; CB^T—variant with cellulolytic bacteria; NB^T—variant with nitrifying bacteria; NFB^T—variant with nitrogen-fixing bacteria. The compost fractions studied were: ≤0.25 mm, 0.5 mm, 1.0 mm, and ≥2.0 mm. Levels of *p*-values: very high (***, $p < 0.001$), high (**, $p < 0.01$), significant (*, $p < 0.05$), not significant (ns, $p \geq 0.05$).

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The effect of cellulolytics in the 0.5 mm and 1.0 mm fractions was noted by a slight increase in total nitrogen, by 5.7% and 3.6%. Under the influence of nitrifiers, there was a tendency for the total nitrogen content to decrease as the fraction size increased. Thus, compared to the control variant, in the 0.5 mm, 1.0 mm, and ≥2.0 mm fractions, nitrogen decreased by 2.8%, 5.4%, and 34.0%, respectively. The control variant relative to the experimental variants shows an excess of total nitrogen content in the ≥2.0 mm fraction. Compared to cellulolytics, nitrifiers, and nitrogen fixers, the difference is 32.7%, 51.4%, and 20.8%, respectively. The fertilizer value for total nitrogen in individual fractions, as in the case of HA, should be assessed taking into account their share in the final compost (Figure 3b).

The total nitrogen content in the compost under the influence of the studied microorganisms varies within the range of 9.1–7.0%, where the minimum content was noted in the variant with nitrifying microorganisms (16.9 kg/t). The maximum (19.73 kg/t) was found in the variant with nitrogen-fixing thermophiles. The total nitrogen content of cellulolytics does not differ from that of the control variant. At the same time, differentiation of nitrogen content by fractions demonstrates more significant differences. The effect of microorganisms on the accumulation of total nitrogen is more pronounced in the smallest fractions (≤0.25 mm and 0.5 mm). Thus, the nitrogen content under the influence of cellulolytics, nitrifiers and nitrogen fixers increases by 6.3% ($p = 0.283$ (not statistically significant)), 12.9% ($p = 0.02$) and 20.0% ($p = 0.001$), respectively, for the fraction ≤ 0.25 mm and by 33.0%, 9.1% and 33.8% for the fraction 0.5 mm.

A large amount of nitrogen accumulates in the 1.0 mm fraction. For this fraction, the maximum effect was observed in the treatments with cellulolytics, 6.58 kg/t, and nitrogen fixers, 6.90 kg/t, which is 12.3% ($p = 0.009$) and 17.8% ($p < 0.001$) higher than in the control. Nitrifiers were less effective for this fraction (5.49 kg/t), representing a 6.3% ($p = 0.158$) reduction. In the largest fraction (≥2.0 mm), the maximum total nitrogen content (4.47 kg/t) was observed in the control, which can be attributed to its lower degree of mineralization. Under the influence of microorganisms, 2.33–2.60 kg ($p < 0.001$) of nitrogen is accumulated in this fraction per 1 ton of compost.

The loss of total nitrogen from the composted mass under the action of nitrifiers is obviously related to the increased denitrification, which is the subsequent stage of nitrogen transformation into gaseous forms. This requires finding ways to limit the loss, such as using nitrification inhibitors, but it is unclear how these substances will affect the overall nitrogen cycle in the compost and the biodegradation process.

The importance of the method for differentiated compost quality assessment by fractions is associated not only with the ability to monitor the intensity and direction of biofermentation processes but also with the ability to more reliably interpret the obtained data. As an example, the correlations between total nitrogen and HA values based on the initial analysis results and after their conversion to gross values, taking into account the proportion of each individual fraction (Table 3), can be seen.

As can be seen from the table, the correlations between total nitrogen and HA values based on relative values do not show a consistent pattern in their content across fractions. Correlation coefficients vary and range from r = −0.765 to r = 0.716.

When converting relative values to absolute values and then taking into account the proportion of each fraction, we obtain highly positive values. The correlation coefficients range from r = 0.902 to 0.976.

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Table 3. Correlations between nitrogen and HA content.

Factions	Control (%)		CB^T (%)		NB^T (%)		NFB^T (%)	
	N	HA	N	HA	N	HA	N	HA
Options (Based on the analysis results)								
≤0.25	1.88	1.87	2.14	1.84	1.97	2.00	2.26	1.97
0.5	1.77	1.93	1.87	1.73	1.72	1.97	1.98	2.09
1.0	1.68	2.27	1.74	1.82	1.59	2.03	1.86	2.02
≥2.0	2.15	2.18	1.62	1.60	1.42	2.12	1.78	1.63
r	0.041		0.716		−0.765		0.468	
Options (In terms of absolute units, taking into account the proportion of fractions)								
≤0.25	4.59	4.56	4.88	4.20	5.18	5.26	5.51	4.81
0.5	3.52	3.84	4.68	4.33	3.84	4.39	4.71	4.97
1.0	5.86	7.92	6.58	6.88	5.49	7.0	6.9	7.49
≥2.0	4.47	4.53	2.33	2.3	2.4	3.58	2.6	2.38
r	0.943		0.976		0.902		0.969	

3.6. The Impact of Microorganisms on the Content of Mineral Nitrogen

One of the main goals of biological fermentation of livestock byproducts is to increase nitrogen availability to plants through the intensive mineralization of nitrogen-containing organic compounds. At the same time, the risk of significant nitrogen loss as ammonia must be considered. The study of various microbial strains is aimed at finding ways to limit non-productive nitrogen losses while improving the overall nitrogen status of compost.

The expediency of introducing selected strains into compostable material is confirmed by test results (Figure 3c).

The proportions of compost fractions when calculating the mineral nitrogen content by physical weight (kg per ton of compost) introduce certain adjustments that more accurately reflect the nitrogen status of each fraction (Figure 3d). In terms of the overall accumulation of mineral nitrogen forms in compost, nitrogen fixers are the most effective, with an accumulation rate of 8.85 kg per ton of compost. This is more than 30% higher than the control (6.79 kg/t). The increase in nitrogen due to cellulolytics is 7.2% (7.28 kg/t). Nitrifiers contribute to a decrease in mineral nitrogen content by 5.3% (6.43 kg/t).

Thermophilic nitrogen-fixing microorganisms had the greatest impact on the accumulation of mineral nitrogen. Thus, with the exception of the 0.5 mm fraction, in the fractions ≤ 0.25 mm, 1.0 mm, and ≥2.0 mm, the increase was 28.1% ($p < 0.001$), 51.1% ($p < 0.001$), and 30.6% ($p < 0.001$), respectively. The cellulolytic thermophilic strain also proved effective, but to a lesser extent. For fractions ≤ 0.25 mm and 1.0 mm fractions, an increase was observed at 15.2% ($p = 0.006$) and 15.9% ($p = 0.002$), respectively, while in the ≥2.0 mm fraction, a decrease of 15.7% ($p = 0.002$) was observed. No significant changes were observed in the ≤0.25 mm fraction under the influence of CB^T and NM^T.

The efficiency of nitrifying microorganisms in terms of mineral nitrogen accumulation is observed in the 0.5 mm fraction, with a change of 14.7% ($p = 0.007$), while in the low-decomposed part, there is a decrease in mineral nitrogen forms by 19.5% ($p < 0.001$) in the 1.0 mm fraction and 11.0% ($p = 0.015$) in the ≥2.0 mm fraction.

Given the narrow functionality of nitrifying microorganisms, it is more accurate to evaluate their effect not through their influence on the total mineral nitrogen content, but on its nitrate form in the compost. In this case, for individual fractions, we see results consistent with their function, specifically the accumulation of nitrate nitrogen and a significant reduction in the ratio with ammonium nitrogen (Figure 4).

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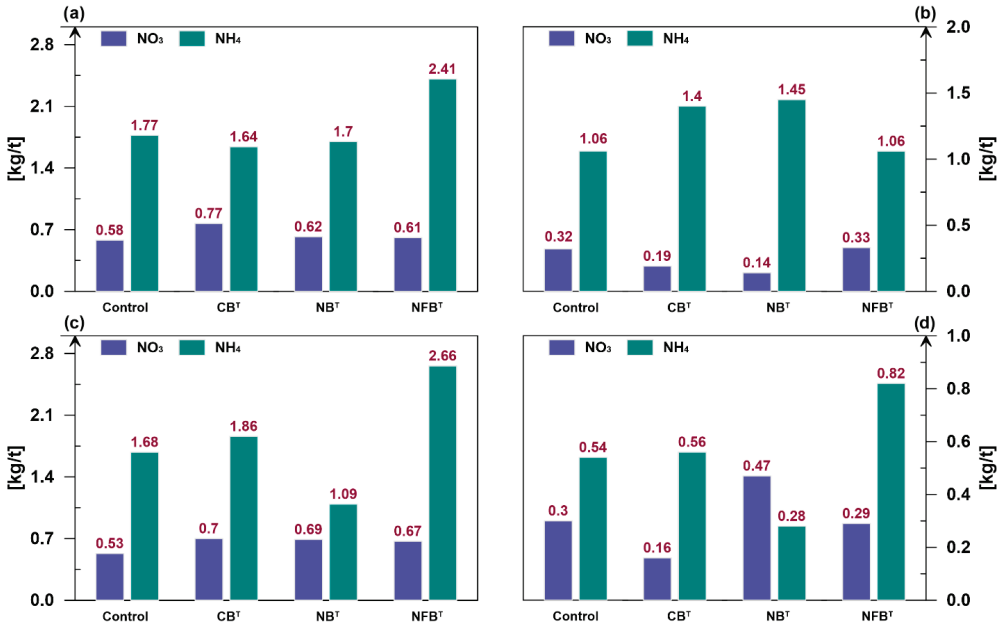

Figure 4. Distribution of mineral forms of nitrogen in compost by fractions: (**a**) ≤0.25 mm; (**b**) 0.5 mm; (**c**) 1.0 mm, and (**d**) ≥2.0 mm. Designations in the figures: NO_3—nitrate and ammonium; NH_4—ammonium; Control—control variant; CB^T—variant with cellulolytic bacteria; NB^T—variant with nitrifying bacteria; NFB^T—variant with nitrogen-fixing bacteria.

Looking at the figure, it should be noted that the ammonium nitrogen content is higher than the nitrate nitrogen content in most variants. On average, nitrogen-fixing microorganisms have the greatest influence on the total ammonium content across fractions. Under their influence, 6.95 kg/t accumulates in the compost, which is 37.6% higher than in the control variant (5.05 kg/ha). Ammonium nitrogen, 5.46 kg/t, accumulates in the compost under the influence of cellulolytic bacteria (+8.1%). The minimum accumulation of ammonium nitrogen (4.52 kg/t) was observed in the variant with nitrifying bacteria, which is 10.5% lower than in the control variant. In terms of nitrate nitrogen, nitrifying microorganisms proved to be the most effective, producing 1.92 kg/t of NO3 in the compost, which is 9.7% higher than the control (1.75 kg/t) and 5.5% higher than the cellulolytic-based treatment (1.82 kg/t). Nitrogen fixers were slightly inferior to nitrifiers in their ability to accumulate nitrate nitrogen in the compost (1.90 kg/t).

A distinctive feature of the nitrifying strain was its activity in larger compost fractions. For example, in the 1.0 mm and ≥2.0 mm fractions, NO_3 accumulation was higher than in the control by 30.2% and 51.6%. In the largest fraction, nitrifiers were significantly more effective than cellulolytics and nitrogen fixers by 193.8% and 62.1%, respectively. It can be assumed that large fractions of compost mass provide a favorable ecological niche for nitrifying activity. This thesis is supported by the narrow ratio of nitrate to ammonium nitrogen. This ratio in the fraction ≥ 2.0 mm is 1.68, while for other variants it is 0.57 (Control), 0.29 (CB^T), and 0.35 (NFB^T).

The influence of nitrifying microorganisms in this case can be assessed as climatically favorable, as it is accompanied by a decrease in the proportion of ammonium, a key predictor of nitrous oxide flux into the atmosphere [32].

Fractional differentiation of the nitrogen status of the compost demonstrates closer correlations when comparing nitrate, ammonium forms, and their sums with the total nitrogen content (Figure 5). The figure shows that recalculating the mineral nitrogen content of compost by fractions more accurately reflects their relationship with the total nitrogen content.

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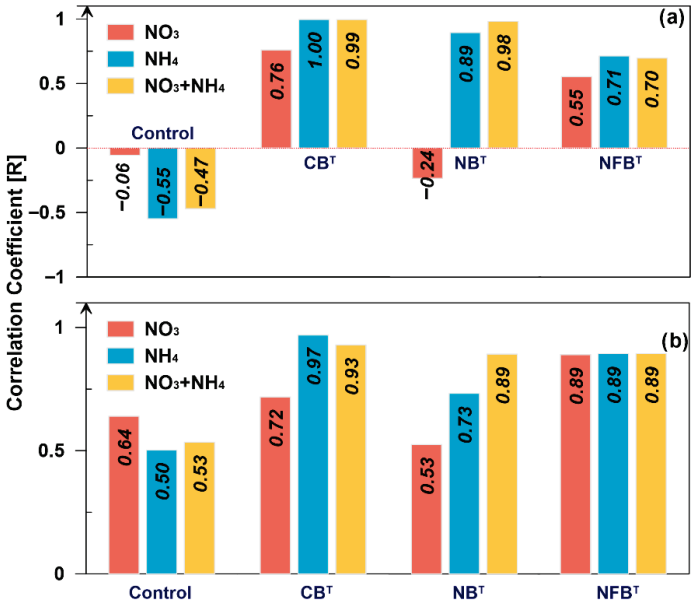

Figure 5. Correlation relationships between mineral forms of nitrogen and total nitrogen in compost (**a**) without and (**b**) with the proportion of fractions. Control—control variant; CB^T—variant with cellulolytic bacteria; NB^T—variant with nitrifying bacteria; NFB^T—variant with nitrogen-fixing bacteria.

3.7. Bioreclamation Properties of Individual Compost Fractions

Biological indicators are increasingly being used as indicators of soil fertility. Given the subject of the study—the influence of microorganisms on the transformation of organic matter from livestock byproducts—microbial biomass carbon and invertase activity were used as bioindicators. Microbial biomass carbon represents an important part of the labile fraction of soil organic matter, responsible for effective fertility [33]. Invertase, in turn, indicates the intensity of organic matter decomposition [34].

The application of compost treated with different microbial strains to soil has different effects on the accumulation of microbial biomass carbon (Figure 6a).

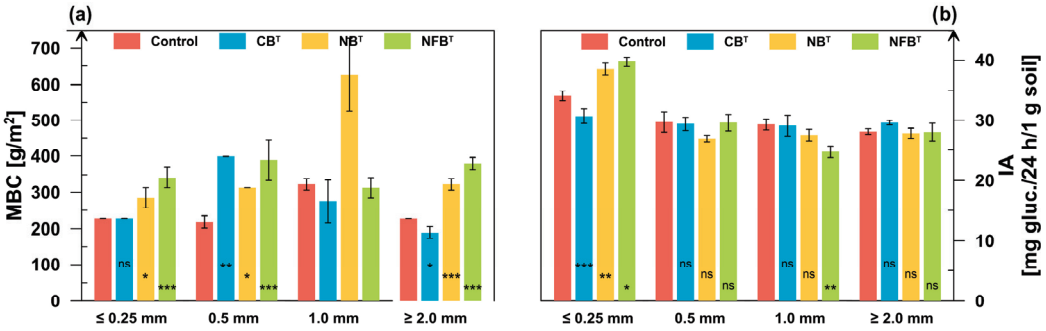

Figure 6. Content of microbial biomass carbon (**a**) and invertase activity in soil fertilized with different fractions of compost (**b**). Designations in the figures: MBC—microbial biomass carbon, g/m^2; IA—invertase activity, mg glucose/24 h/1 g of soil; Control—control variant; CB^T—variant with cellulolytic bacteria; NB^T—variant with nitrifying bacteria; NFB^T—variant with nitrogen-fixing bacteria. The compost fractions studied were: ≤0.25 mm, 0.5 mm, 1.0 mm, and ≥2.0 mm. Levels of *p*-values: very high (***, $p < 0.001$), high (**, $p < 0.01$), significant (*, $p < 0.05$), not significant (ns, $p \geq 0.05$).

Thus, under the influence of the smallest compost fraction (≤0.25 mm), the maximum accumulation of microbial biomass carbon is observed in the soil variant using thermophilic nitrogen fixers (342 g/m^2), which is 50.0% ($p < 0.001$) higher than in the control variant (228 g/m^2). Nitrifiers increase this indicator by 25.0% (285 g/m^2) ($p = 0.03$). The Microbial

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Biomass Carbon (MBC) content under the influence of cellulolytics is equivalent to that in the control variant.

Under the influence of the 0.5 mm compost fraction, all studied strains (NFB^T, NB^T, CB^T) increase the MBC content by 78.4% ($p = 0.001$), 43.6% ($p = 0.04$), and 83.0% ($p < 0.001$), respectively.

Under the influence of the 1.0 mm compost fraction, there are no statistically significant changes. The coarse compost fraction ($\geq$2.0 mm) obtained with nitrogen fixers and nitrifiers increased MBC by 66.7% ($p < 0.001$) and 41.7% ($p < 0.001$), respectively. Cellulosolytics, on the other hand, reduced microbial carbon content by 16.7% ($p = 0.04$).

Summarizing the assessment of compost bioreclamation properties based on microbial biomass carbon, it can be concluded that nitrogen fixers and nitrifiers in compost, in most cases, act as activators of the accumulation of labile organic matter in the soil.

Changes in soil invertase activity under the influence of biofermented compost are observed mainly with the addition of the smallest fraction (Figure 6b). The highest invertase activity is observed in soil samples fertilized with the smallest compost fraction ($\leq$0.25 mm). This trend applies to all experimental variants, but with different degrees. When examining the effects of the treatments within a single fraction of $\leq$0.25 mm, it can be noted that the use of nitrogen-fixing and nitrifying strains in biofermentation increases the bioremediation properties of compost by 16.4% ($p < 0.001$) and 12.9% ($p < 0.002$), respectively. Conversely, the treatment with cellulolytics reduces soil invertase activity by 10.0% ($p = 0.011$).

Differences in the effects of microorganisms on invertase activity in larger fractions of 0.5 mm, 1.0 mm, and $\geq$2.0 mm narrow and range from 26.9 to 29.7, 24.7–29.3, and 27.8–29.6 mg glucose/24 h/1 g soil, respectively.

4. Conclusions

The results of the study show that the use of bioagents (target microbial strains) can influence the direction of transformation processes in biocompostable materials, improving the quality of the final product—the degree of fragmentation (destruction), humification, and nitrogen balance, which ultimately impacts the bioreclamation properties of the compost.

The study also suggests that compost fractionation can be considered as a promising method for assessing the quality of poultry manure biofermentation, where the proportion of the smallest fractions reflects the rate of degradation of the primary raw material. Thus, the rate of degradation (fragmentation) of the compost depended on the microorganisms used. In the 0.5 mm fraction, all compared strains were effective, with the maximum degradation properties, 25.6% ($p = 0.0024$), demonstrated by cellulolytic microorganisms.

The total HA content in the $\leq$0.25 mm fractions increased by 15.4% ($p = 0.01$) under the influence of nitrifiers. Under the influence of nitrifies, the increase in 0.5 mm was 14.3% ($p = 0.03$). Nitrogen-fixers were more effective only in the 0.5 mm fraction, whose share increased by 29.4% ($p < 0.001$).

The studied microorganisms also had a positive effect on the nitrogen balance in the compost. Thus, with a relatively small spread in total nitrogen content in the compost across treatments: 18.44 kg/t (Control), 18.47 kg/t (CB^T), 16.91 kg/t (NB^T), and 19.27 kg/t (NFB^T), its redistribution into the fine fractions was observed under the influence of the microorganisms. Thus, in the $\leq$0.25 mm fraction, the total nitrogen content increases by 6.3% (CB^T) ($p = 0.283$), 12.9% (NB^T) ($p = 0.02$), and 20.0% (NFB^T) ($p = 0.001$). Under the influence of the same microorganisms, the total nitrogen content in the 0.5 mm fraction increases by 33.0% ($p < 0.001$), 9.1% ($p = 0.04$), and 33.8% ($p < 0.001$), respectively.

Methodologically, it was more appropriate to calculate the total content of HA and various forms of nitrogen, taking into account the fraction distribution. This was established

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by the correlation coefficients between the analytical parameters expressed in g/kg compost and the total content, recalculated per kg/t compost, taking into account the fraction proportions. Thus, in the first case, the spread of the correlation coefficients across the variants was r = 0.041 (Control), r = 0.716 (CB^T), r = −0.765 (NB^T), and r = 0.468 (NFB^T). When taking into account the total nitrogen content, the correlation coefficients in the same variants were: r = 0.943, r = 0.976, r = 0.902, and r = 0.969, respectively. A similar pattern was found when comparing the sum of mineral nitrogen forms with their total nitrogen content. In the first case, the correlation coefficients did not follow a consistent pattern and were: r = −0.06 (Control), r = 0.760 (CB^T), r = −0.24 (NB^T), and r = 0.55 (NFB^T). When considering the total nitrogen content, the correlation coefficients were positive, with medium and high correlation levels: r = 0.64 (Control), r = 0.720 (CB^T), r = 0.530 (NB^T), and r = 0.890 (NFB^T).

The use of microorganisms in the fermentation of poultry manure also increased the bioremediation potential of the fine fractions of the compost. The increase in soil invertase activity and microbial biomass carbon accumulation increased by 16.4% ($p < 0.001$) and 50% ($p < 0.001$), respectively, with the addition of the minimum fraction ($\leq$0.25 mm) of compost processed with nitrogen fixers. These parameters increased by 12.9% ($p = 0.02$) and 25.0% ($p = 0.03$), respectively, with nitrifiers. The maximum effect of cellulolytics—83.0% ($p < 0.001$) on the growth of MBC in soil was achieved with the addition of the 0.5 mm compost fraction.

Prospects for further research include choosing the sequence for adding the isolated strains to the composted mass and determining the optimal time intervals between microbial applications.

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Abbreviations

The following abbreviations are used in this manuscript:

CB^T	Thermophilic Cellulolytic Bacteria
NB^T	Nitrifying Bacteria
NFB^T	Nitrogen-Fixing Non-Associative Bacteria
rRNA	Ribosomal ribonucleic acid
HA	Humic acids
MBC	Microbial biomass carbon

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Article

Survival of Bacterial Pathogens During Storage of Animal Waste and Wastewater Treatment Sludge and Their Subsequent Application to Clay–Loam Soil

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Abstract

Globally, large quantities of animal waste and human sewage sludge are generated annually. Their application as soil amendments can enhance soil quality and support a circular economy. However, these wastes may harbour pathogenic bacteria, posing contamination risks to soil and water and potential transmission to animals and humans. This study investigated the survival of five bacterial pathogens during six months of storage in five types of organic waste and following their subsequent application to soil. During storage, T_{90} values ranged as follows: *Salmonella* Typhimurium (2.3–17.7 days), *Campylobacter jejuni* (0 to 23.9 days), *Escherichia coli* O157:H7 (4.3 to 57.8 days), and *Listeria monocytogenes* (1.9 to 170.4 days). In soil, T_{90} values were *S.* Typhimurium (4.2 to 17.4 days), *C. jejuni* (4.8 to 26.8 days), *E. coli* O157:H7 (4.3 to 52.9 days), and *L. monocytogenes* (2 to 83.7 days). *Clostridium sporogenes* remained stable throughout both experiments, preventing T_{90} calculation. Contrary to our initial hypothesis that soil microbiota would accelerate pathogen decline, T_{90} values were higher during storage in 11 cases and higher in soil in nine scenarios. These findings highlight the need for pre-treatment strategies for animal waste and biosolids before land spreading to consistently mitigate risks of pathogen transmission and environmental contamination.

Keywords: manure; biosolids; storage; soil; bacterial pathogens; survival

1. Introduction

Due to the global increase in population, millions of tonnes of agricultural, municipal, and industrial waste are generated annually. In Ireland, the Agriculture, Forestry, and Fishing sector has expanded by 85% over the past 30 years, contributing to a Gross Value Added (GVA) of EUR 4,501 million in 2024 [1]. This growth is supported by 4678 hectares of agricultural land [2] and a livestock population comprising 7,183,100 cattle, 5,175,700 sheep, and 1,679,900 pigs [3]. Globally, approximately 13 billion tonnes of animal waste are produced each year [4]. In Ireland alone, 69,419 tonnes of dry human sewage sludge were generated in 2024, all of which was reused as soil fertiliser after appropriate treatment [5].

The EU Green Deal promotes a circular economy by re-using animal waste. Consequently, applying organic waste as fertiliser improves soil quality, as it provides essential nutrients and metals, stabilises the organic matter content and the carbon to nitrogen (C/N) ratio, and enhances buffer capacity and cation exchange [6–8]. Moreover, they are readily

available with minimal or no cost to the farmer. Various technologies are used nowadays to sanitise waste, minimise odour, reduce volume, and lower nutrient content Storing manure is a cost-effective and convenient method for farmers before land application [6]. Liquid or slurry fractions are typically kept in slurry pits or tanks, whereas solid manure and poultry waste are stored in heaps [7]. To prevent the risk of runoff and contamination of adjacent grazing fields or watercourses, spreading animal manure is not permitted in Ireland from November until the end of January. A minimum storage period for cattle manure has been established, ranging from 16 to 22 weeks depending on the geographical area [9].

Recycling organic wastes as a soil fertiliser is regulated by Regulation 1069/2009 and the Waste Management Act of 1996 [10,11]. In addition, the European Commission (EC) Directive 2018/850 requires a reduction in municipal waste landfilled to 10% or less by 2035, to reduce greenhouse gas (GHG) emissions and the pollution of surface water, groundwater, soil, and air [12]. Hence, the EU Green Deal aims to cut GHG emissions by 50% by 2035 [13]. Specifically, agriculture is responsible for 37.8% of the total GHG emissions in Ireland [14].

Animal and human waste may harbour pathogenic bacteria that pose a risk to public and animal health. The gastrointestinal tracts of poultry, swine, and cattle serve as primary reservoirs of *Campylobacter jejuni*, where concentrations can reach up to 10^8 colony-forming units (CFU) per gram [15,16]. *Salmonella* spp. and *Escherichia coli* O157:H7 may be found at similar concentrations in the intestine of humans and warm-blooded animals [16,17]. Furthermore, *Listeria monocytogenes* and members of the *Clostridium* genus, including *Clostridium botulinum* or *Clostridium perfringens*, are widely spread in the environment [16,18]. Factors such as temperature of storage, dryness, concentration of solids, pH or volatile fatty acids (VFA), or ammonia concentration have been described as the main causes of pathogen die-off in manure [19]. While storage can lead to some decline in bacterial levels, pathogens can proliferate if nutrient availability is sufficient, particularly with the addition of fresh manure [20–23]. Hence, the survival of bacterial pathogens in soil is influenced by factors such as temperature, pH, oxygen levels, moisture, UV-radiation, and autochthonous microbiota [20,24].

The application of animal manure and biosolids to agricultural land can result in soil contamination, thereby facilitating the transfer of these pathogens to vegetables and crops [25]. Furthermore, surface water may become contaminated by bacterial pathogens originating from organic wastes. These pathogens can migrate into groundwater during rainfall events, leading to surface runoff and elevating the risk of waterborne disease outbreaks, ultimately contributing to the microbial pollution of natural ecosystems [26,27].

Additionally, the use of animal manure and biosolids promotes the dissemination of antibiotic-resistant genes (ARG) to environmental bacterial populations and soil [28]. In 2019, bacterial antimicrobial resistance (AMR) was linked to roughly 1.27 million deaths worldwide as a direct cause and contributed to an additional 4.95 million fatalities [29]. Consequently, the spread of antimicrobial resistance poses a significant and growing threat to global public health.

Enteric pathogens such as *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* have been shown to persist for several months in manure-amended soil [23,30], whereas *C. sporogenes* can remain stable for years [31,32]. The presence of pathogenic microorganisms in agricultural soil poses a significant risk to the safety of fresh produce, particularly as these products are often consumed raw or after minimal processing [25]. In 2023, 40 foodborne outbreaks (FBO) were attributed to the consumption of contaminated fresh produce in the EU. The majority of these outbreaks were linked to *Salmonella* spp. (seven FBO), enterotoxigenic *E. coli* (one FBO involving 154 reported cases), *L. monocytogenes* (one FBO) and the toxins of *C. perfringens*, among others [33].

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Given all the potential risks posed to environmental, human, and animal health, the aim of this study was to determine the survival of *S.* Typhimurium, *C. jejuni*, *E. coli* O157:H7, *L. monocytogenes*, and *C. sporogenes* (used as surrogate for *C. botulinum*) during the storage of animal wastes and wastewater treatment sludge over six months. The studies were conducted under average Irish summer and winter temperatures, as well as following land application, with the objective of determining whether pre-treatment of the aforementioned wastes is necessary prior to land spreading.

2. Materials and Methods

2.1. Sample Collection

Fresh animal waste samples were collected in October 2024 from various locations in Ireland. Cattle and swine slurry were collected from manure pits in County Meath and County Cork, respectively. Fresh sheep samples were taken directly from grazing fields in County Cavan, while broiler faecal samples were taken from inside the growing houses in County Monaghan. The wastewater treatment sludge (WWTS) was sourced in Ireland. Clay–loam soil was extracted from Teagasc Oak Park Crops Research Centre (Oak Park, Carlow, Dublin) and stones were removed by sieving.

2.2. Inocula Preparation

Samples were inoculated individually with reference strains: *Salmonella* Typhimurium ATCC 13311, *Listeria monocytogenes* ATCC 35152, *Escherichia coli* O157:H7 EDL931, *Campylobacter jejuni* ATCC 700819, and *Clostridium sporogenes* DSM 767. They were obtained from the culture collection in Teagasc Food Research Centre (Ashtown, Dublin, Ireland). The inoculum preparation protocol for *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 was adapted with slight modifications from Russell et al. (2022), as was the spore preparation [32], while the protocol for *C. jejuni* was adapted from Meredith et al. (2013) [34].

Briefly frozen stocks on cryoprotective beads of *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 were streaked on Tryptone Soya Agar (TSA; Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland) and incubated at 37 °C for 24 h. Then, a single colony was transferred to 10 mL of Brain Heart Infusion (BHI; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) and incubated overnight at 37 °C. The culture was centrifuged at 8014× *g* for 10 min, washed, and resuspended in phosphate-buffered saline (PBS; SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland). The final concentration of the cultures was approximately 8 $\log_{10}$ CFU/mL.

One bead of *C. jejuni* was placed in Hunts Broth that contained 0.65 g nutrient broth (Oxoid, Fisher Scientific Ltd., Dublin, Ireland), 0.15 g Yeast Extract (Oxoid, Fisher Scientific Ltd., Dublin, Ireland), 5% Lysed Horse Blood (Cruinn diagnostics, Dublin, Ireland), and 0.4% *Campylobacter* Growth Supplement (SR232E, Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland) [35]. The broth was incubated at 42 °C for 48 h in microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) (Oxoid, Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland). Exactly 1 mL of the culture was then inoculated in 100 mL Hunts broth and 42 °C for 24 h in microaerobic conditions. Cells were harvested by centrifuging at 3214× *g* for 10 min, and washing three times with Maximum Recovery Diluent (MRD; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) [34].

100 µL of rehydrated *C. sporogenes* culture was added to 20 tubes each containing 20 mL of cooked meat medium (CMM; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) and incubated in an anaerobic chamber for 12–18 h at 37 °C. Aliquots of 300 µL of the broth were spread on 300 plates of *Clostridium* Sporulation Agar (CSA: tryptone 5 g, bacteriological peptone 5 g, lab lemco 1 g, yeast extract 2 g, $CaCl_2$ 0.056 g, $MnSO_4$:$4H_2O$ 0.082 g, glucose

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1 g, agar 15 g; made up to one litre with deionised water) [36] and incubated in anaerobic conditions (Anaerogen Sachets, Oxoid, Fannin Ltd., Galway, Ireland) at 37 °C for 12 days. Approximately 5 mL of ice-cold sterile water was added on the CSA plates and agitated with a spreader to release the spores from the surface. The remaining suspension was pooled in 50 mL tubes and subsequently centrifuged at 7500× *g* for 10 min at 4 °C and washed three times with ice-cold sterile water, reducing the amount of water by 5 mL each time [32]. A concentration of approximately 10^7 spores/mL was added to Reinforced *Clostridium* Agar (RCA; Oxoid, Fisher Scientific Oxoid Ltd., Dublin, Ireland) in anaerobic conditions, after a heat treatment of 80 °C for 10 min.

2.3. Sample Inoculation and Incubation

For the storage study, each bacterium of interest was added to 50 g of waste in triplicate, while the soil samples were prepared on a 1:1 soil-to-waste ratio. All samples were kept in 250 mL Ramboldisterile containers (Sparks Lab Supplies, Rathcoole, Dublin, Ireland).

A total of 300 samples were prepared and individually inoculated, reaching a final concentration of 6 $\log_{10}$ CFU/g or spores/g. Half were incubated at 4 °C and the remainder at 14 °C, which represented the average winter and summer temperatures in Ireland.

2.4. Physico–Chemical and Microbiological Analyses

The pH was determined using a FiveGo Portable pH Meter (Mettler Toledo, Columbus, OH, USA), while the water activity (a_w) was assessed with an Aqualab Pre Water Activity Meter (Labcell, Alton, UK) at each sampling point.

The bacterial concentration of the samples from the storage study were analysed every two weeks, while the soil samples were measured weekly during the first month and every month up to 168 days thereafter, or until two consecutive negative enrichments were observed.

A 5 g sample was extracted from each container and diluted in 45 mL of the sterile corresponding broth and homogenised for 90 s using a Star Blender LB 400 Stomacher (VWR, Lutterworth, Leicestershire, UK). Serial dilutions were prepared and plated in selective media. Protocols for microbiological enumeration and detection of *S. enterica*, *E. coli* O157:H7, *L. monocytogenes*, and *C. sporogenes* were described by Russell et al. (2022), while ISO method 10272 was used for *C. jejuni* [32,37].

Detection methods were employed when pathogen concentrations fell below the limit of detection. For *S. enterica*, 5 g of sample were diluted in 45 mL of Buffered Peptone Water (BPW; Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland) and incubated at 37 °C for 24 h. Subsequently, 100 µL of the culture was distributed in three drops on Modified Semi-Solid Rappaport Vassiliadis medium (MSRV; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) supplemented with Novobiocin supplement (20 mg/L, Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland) and incubated at 42 °C for 24 h. Colonies exhibiting a halo were streaked onto XLD and incubated at 37 °C for 24 h for confirmation [32].

For *E. coli* O157:H7, enrichment was performed by incubating 5 g of sample in modified Tryptone Soya Broth (mTSB; Oxoid, Dublin, Ireland) supplemented with cefixime (50 µg/L) and vancomycin (6 mg/L) at 42 °C for 24 h. Cultures were then streaked onto CT-SMAC [32].

Detection of *C. jejuni* involved adding 5 g of sample to 45 mL of Bolton Broth (Oxoid, Fisher Scientific Ltd., Dublin, Ireland), followed by incubation at 42 °C for 48 h under microaerobic conditions. Aliquots of 100 µL were plated on mCCDA and incubated under the same conditions [37].

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For *L. monocytogenes*, 5 g of sample were added to 45 mL of half-strength Fraser Broth (Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland) supplemented with Half Fraser Supplement (SR0166E; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) and incubated overnight at 30 °C. Then, 100 µL of the culture was added to 10 mL of full-strength Fraser broth (SLS Scientific Laboratory Supplies, Dublin, Ireland) supplemented with Fraser Supplement (SR0156E; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) and incubated at 37 °C for 48 h. An aliquot of 100 µL of the culture was plated onto *Listeria* Selective Oxford Agar [32].

For the enumeration of target pathogens, a 5 g sample was extracted from each container and diluted in 45 mL of MRD. The sample was homogenised for 90 s using a Colworth Stomacher 400, followed by serial tenfold dilutions in MRD. Aliquots of 100 µL were plated on selective media appropriate for each pathogen: *S. enterica* was cultured in Xylose Lysine Deoxycholate (XLD; Oxoid, Fisher Scientific Ltd., Dublin, Ireland); *E. coli* O157:H7 on Sorbitol MacConkey agar (SMAC; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) supplemented with cefixime-tellurite (SR0172E; Oxoid, Fisher Scientific Ltd., Ireland), *C. jejuni* onto modified *Campylobacter* Blood-Free Selective Agar Base (mCCDA; Oxoid, Fisher Scientific Ltd., Dublin, Ireland) supplemented with CCDA Selective Supplement (SR0155E; Oxoid, Fisher Scientific Ltd., Dublin, Ireland), *L. monocytogenes* on *Listeria* Selective Oxford Agar Base (Oxoid, Fisher Scientific Ltd., Dublin, Ireland) supplemented with *Listeria* Selective Supplement Oxford (SR206E, Oxoid, SLS Scientific Laboratory Supplies Ltd., Dublin, Ireland), and *C. sporogenes* on RCA.

2.5. Data Analysis

As previously mentioned, the samples were prepared in triplicate and bacterial counts were converted into $\log_{10}$ CFU/g and the mean calculated. The T_{90} value is the time required to obtain a 90% reduction in the microbial population (1 $\log_{10}$), and it was determined by linear regression using Graphpad Prism 10 software (San Diego, CA, USA) [38]. For each study, two-way ANOVA and Tukey's Honestly Significant Difference (HSD) post hoc tests were conducted to assess the effect of waste type and temperature on bacterial survival. Furthermore, statistical differences between the T_{90} values of storage and soil experiments for a given bacteria, waste and temperature were calculated using paired *t*-tests. All statistical analyses were conducted using R 4.5.0 (R Core Team, 2024) and statistical significance was set at $p < 0.05$ [39].

3. Results

3.1. pH and a_w

The pH in the bovine slurry, ovine slurry, pig slurry, poultry litter, and WWTS ranged from 6.7 to 8.0, 7.5 to 8.2, 8.1 to 8.8, 8.7 to 9.3, and 6.7 to 8.2, respectively, regardless of storage temperature, while the corresponding values in amended soil were 7.8 to 8.0, 7.5 to 8.1, 8.2 to 7.7, 7.7 to 9.3, and 7.0 to 9.0, respectively. The a_w in all samples was approximately 0.99, except for poultry samples that ranged from 0.93 to 0.98.

3.2. Storage Study

Maximum survival time in days during storage are presented in Table 1 and T_{90} values for the storage experiment are provided in Table 2. Briefly, the survival of *S.* Typhimurium and *C. jejuni* ranged from 0 to 5 months, *E. coli* O157:H7 from 21 days to more than six months, and lastly, *L. monocytogenes* and *C. sporogenes* were viable after six months.

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Table 1. Maximum survival in days of the five target bacteria in the storage and soil experiments.

Sample	T (°C)	Bovine Waste		Ovine Waste		Pig Waste		Poultry Waste		WWTS	
		Waste	Soil	Waste	Soil	Waste	Soil	Waste	Soil	Waste	Soil
S. Typhimurium	4	84 (1) [1]	56 (2)	28 (1)	56 (3)	28 (2)	56 (1)	56 (2)	28 (2)	84 (1)	84 (1)
	14	56 (2)	56 (1)	56 (1)	56 (1)	14 (1)	28 (2)	14 (1)	56 (2)	56 (3)	56 (2)
E. coli O157:H7	4	>168 [2] (3)	>168 (3)	>168 (3)	>168 (3)	84 (3)	112 (1)	21 (3)	84 (3)	>168 (3)	>168 (3)
	14	>168 (3)	84 (3)	>168 (3)	>168 (3)	84 (2)	84 (2)	21 (1)	84 (2)	>168 (3)	140 (3)
C. *jejuni*	4	0 (3)	14 (1)	112 (3)	140 (3)	0 (3)	56 (3)	140 (3)	140 (1)	140 (3)	140 (3)
	14	0 (3)	28 (3)	140 (3)	140 (1)	0 (3)	56 (3)	140 (3)	140 (1)	140 (3)	140 (3)
L. monocytogenes	4	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)
	14	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)
C. sporogenes	4	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)
	14	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)	>168 (3)

[1] "()" denotes the number of replicates that were positive by enrichment. [2] ">" denotes that the sample was positive after six months of storage.

Table 2. T_{90} values for the different bacteria when waste is inoculated and incubated at 4 and 14 °C.

Bacteria	Waste	Incubation at 4 °C					Incubation at 14 °C				
		R^2	Slope	SE	*n*	T_{90} (Days)	R^2	Slope	SE	*n*	T_{90} (Days)
S. Typhimurium	Bovine	0.85	−0.072	0.0074	18	13.9	0.88	−0.070	0.0065	18	14.3 *
	Ovine	0.80	−0.206	0.0394	9	4.9 *,†	0.99	−0.425	0.0185	6	2.4 *,†
	Pig	0.85	−0.124	0.0165	12	8.1 †	0.85	−0.212	0.0340	9	4.7 †
	Poultry	0.71	−0.097	0.0174	15	10.3 †	0.98	−0.437	0.0309	6	2.3 †
	WWTS	0.78	−0.057	0.0075	18	17.7 *	0.82	−0.056	0.0066	18	17.7
E. coli O157:H7	Bovine	0.89	−0.021	0.0015	27	48.8 †	0.79	−0.028	0.0029	27	35.7 *,†
	Ovine	0.84	−0.017	0.0016	24	57.8	0.81	−0.025	0.0055	24	39.6
	Pig	0.86	−0.055	0.0055	18	18.2	0.86	−0.055	0.0055	18	18.2
	Poultry	0.99	−0.234	0.0087	9	4.3	0.99	−0.234	0.0087	9	4.3
	WWTS	0.95	−0.043	0.0023	21	23.2	0.79	−0.047	0.0055	21	21.5 *
C. jejuni	Bovine					0.0 *					0.0 *
	Ovine	0.79	−0.043	0.0047	24	23.5	0.70	−0.049	0.0068	24	20.4 *
	Pig					0.0 *					0.0 *
	Poultry	0.66	−0.047	0.0085	18	21.2 †	0.83	−0.061	0.0070	18	16.4 *,†
	WWTS	0.76	−0.042	0.0054	21	23.9	0.79	−0.042	0.0049	21	23.8
L. monocytogenes	Bovine	0.72	−0.011	0.0016	21	88.3 *	0.59	−0.008	0.0017	18	121.0 *
	Ovine	0.84	−0.022	0.0022	21	45.1 *	0.62	−0.017	0.0030	21	59.6
	Pig	0.50	−0.006	0.0013	21	170.4 *,†	0.93	−0.045	0.0030	18	22.1 *,†
	Poultry	0.99	−0.524	0.0045	6	1.9 †	0.79	−0.256	0.0502	9	3.9 *,†
	WWTS	0.97	−0.079	0.0040	15	12.7 *	0.81	−0.080	0.0109	15	12.5 *

* denotes a statistical difference between the T_{90} values in the storage and soil studies for a given bacteria, waste and temperature of incubation at a 5% level ($p < 0.05$). † denotes a statistical difference between T_{90} values at different temperatures for a given bacteria and waste at a 5% level ($p < 0.05$).

At 4 °C, the lowest T_{90} values observed for *S.* Typhimurium were in ovine (4.9 days) and pig waste (8.1 days), while it increased to 17.7 days in WWTS. *Salmonella* was no longer detectable in ovine and pig waste following one month of storage, in poultry waste after two months, and in bovine waste and WWTS after three months. A similar trend was noted at 14 °C, where T_{90} values ranged from 2.3 to 4.7 days in poultry, ovine, and pig waste, and increased to 14.3 and 17.7 days in bovine and WWTS, respectively. Under these conditions, *Salmonella* viability was lost within one month in poultry and pig waste, two months in ovine and bovine faeces, and three months in WWTS. The greatest impact of storage on the survival of *Salmonella* at 4 and 14 °C was observed in WWTS, followed by bovine waste. At 4 °C, the order of effect was poultry, pig, and ovine waste, with only the difference between pig and ovine waste being statistically significant. At 14 °C, the ranking shifted to pig, ovine, and poultry waste, and again, only the difference between the latter two was significant.

A 90% reduction in *E. coli* O157:H7 was achieved in poultry, pig, and WWTS in 4.3, 18.2, and 23.2 days, respectively, regardless of the incubation temperature. In contrast, a greater difference was observed between 4 and 14 °C in bovine and ovine slurry where

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the corresponding values were 48.8 and 35.7 and 57.8 and 39.6, respectively. *E. coli* was not detected after 21 days of storage in poultry waste and 85 days in pig waste. However, it remained detectable after six months in WWTS, bovine, and ovine wastes. During storage, the interaction between temperature and waste type was not statistically significant for *E. coli* O157:H7. The greatest effect was observed in ovine and bovine samples ($p > 0.05$), followed by WWTS and pig ($p > 0.05$), with poultry waste showing the lowest effect.

C. jejuni was not detected immediately after the inoculation in pig and bovine waste, indicating a rapid die-off or inhibition of the strain. At 4 °C, T_{90} values were slightly higher than at 14 °C in poultry (21.2 and 16.4 days), ovine (23.5 and 20.4 days), and WWTS (23.9 and 23.8 days). Regardless of incubation temperature, *C. jejuni* was no longer detectable after four months in ovine waste and five months in poultry and WWTS. During storage, *C. jejuni* exhibited significantly higher T_{90} values at both 4 and 14 °C in WWTS, ovine, and poultry wastes compared to bovine and pig wastes.

A temperature-dependent difference in *L. monocytogenes* reduction was observed in pig waste, with T_{90} values of 170.4 days at 4 °C and 22.1 days at 14 °C. In contrast, the reduction was greater at 4 °C than at 14 °C in poultry (1.9 and 3.9 days), ovine (45.1 and 59.6 days), and bovine waste (88.3 and 121.0 days), while it was very similar in WWTS (12.7 and 12.5 days). However, *L. monocytogenes* was still viable in all the samples after six months. The interaction between waste type and temperature was statistically significant during storage. At 4 °C, the highest effect was observed in pig waste, followed by bovine, ovine, WWTS, and poultry wastes.

The concentration of *C. sporogenes* remained stable throughout six months of storage regardless of the incubation temperature, indicating the notable persistence of the spores. Since a 90% reduction was not achieved, T_{90} values could not be calculated.

3.3. Bacterial Survival in Waste–Amended Soil

T_{90} values of the target bacteria when organic wastes were applied to clay–loam soil and incubated 4 and 14 °C are shown in Table 3. A similar trend was found in soil, *S*. Typhimurium survived from 14 days to three months, *C. jejuni* from 14 days to five months, and *E. coli* O157:H7 from 21 days to more than six months. Again, *L. monocytogenes* and *C. sporogenes* were detected after more than six months in soil.

At 4 °C, T_{90} values for *S*. Typhimurium ranged from 5.5 days in poultry waste to 9.4 and 10.3 days in pig and bovine waste, respectively, increasing to 14.9 and 17.4 days in WWTS and ovine waste. S. Typhimurium was no longer detectable after one month in poultry, two months in pig and bovine waste, and three months in ovine and WWTS. At 14 °C, the T_{90} values were 4.2, 5.4, 6.1, and 6.4 days for pig, bovine, ovine, and poultry, respectively, and 15.8 days in WWTS. Similarly, the pathogen was undetectable after one month in soil amended with pig waste, two months in bovine, ovine, and poultry, and three months in WWTS. In this case, ovine waste exhibited the highest T_{90} values at 4 °C, followed by WWTS, bovine, pig, and poultry waste. All interactions were statistically significant except for those between bovine and pig wastes ($p > 0.05$). At 14 °C, WWTS had the highest effect, and only comparisons involving this waste were statistically significant ($p < 0.05$).

In soil amended with poultry litter, T_{90} value for *E. coli* O157:H7 was 4.3 days at 4 °C and 14 °C, and it was undetectable after three months of storage. The corresponding values at 4 °C for pig, ovine, WWTS, and bovine were 20.7, 34.5, 36.4, and 52.9 days, respectively. *E. coli* O157:H7 was no longer viable after four months in soil amended with pig waste; however, it remained detectable after six months in soil amended with ovine, WWTS, and bovine waste. At 14 °C, T_{90} values were 14.8 (WWTS), 18.2 (pig), 19.8 (bovine) and 22.3 days (ovine). Under these conditions, *E. coli* O157:H7 was undetectable after three

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months in pig and bovine-amended soil and four months in WWTS-treated soil, though it was viable beyond six months in ovine-amended soil. The statistical analysis revealed a significant interaction between temperature and waste type. Bovine waste had the most pronounced effect on pathogen reduction, followed by WWTS, ovine, pig, and poultry wastes. However, at 14 °C, no significant differences were observed among the waste types.

Table 3. T_{90} values for the different bacteria when waste is applied to soil and incubated at 4 and 14 °C.

Bacteria	Waste Applied	Incubation at 4 °C					Incubation at 14 °C				
		R^2	Slope	SE	*n*	T_{90} (Days)	R^2	Slope	SE	*n*	T_{90} (Days)
S. Typhimurium	Bovine	0.84	–0.097	0.0085	27	10.3	0.85	–0.185	0.0196	18	5.4 *,†
	Ovine	0.79	–0.058	0.0067	21	17.4 *	0.94	–0.164	0.0126	12	6.1 *,†
	Pig	0.81	–0.106	0.0110	24	9.4	0.91	–0.238	0.0209	15	4.2 †
	Poultry	0.89	–0.181	0.0157	18	5.5	0.66	–0.156	0.0281	18	6.4
	WWTS	0.78	–0.067	0.0072	27	14.9 *	0.81	–0.063	0.0058	30	15.8
E. coli O157:H7	Bovine	0.88	–0.019	0.0014	27	52.9 †	0.83	–0.050	0.0053	21	19.8 *,†
	Ovine	0.84	–0.029	0.0027	24	34.5	0.85	–0.044	0.0043	21	22.3
	Pig	0.88	–0.048	0.0040	21	20.7	0.86	–0.055	0.0054	18	18.2
	Poultry	0.99	–0.234	0.0087	9	4.3	0.99	–0.234	0.0087	9	4.3
	WWTS	0.79	–0.028	0.0030	24	36.4	0.85	–0.068	0.0064	21	14.8 *
C. jejuni	Bovine	0.63	–0.209	0.0510	12	4.8 *,†	0.56	–0.102	0.0248	15	9.8 *,†
	Ovine	0.69	–0.047	0.0063	27	21.1 †	0.50	–0.037	0.0075	27	26.8 *,†
	Pig	0.77	–0.103	0.0158	15	9.7 *	0.36	–0.054	0.0179	18	18.7 *
	Poultry	0.54	–0.053	0.0104	24	19.0	0.63	–0.046	0.0076	24	21.7 *
	WWTS	0.82	–0.041	0.0036	30	24.4	0.77	–0.037	0.0041	27	26.6
L. monocytogenes	Bovine	0.80	–0.172	0.0272	12	5.8 *	0.88	–0.188	0.0217	12	5.3 *
	Ovine	0.44	–0.012	0.0027	27	83.7 *,†	0.81	–0.025	0.0023	30	40.4 †
	Pig	0.98	–0.486	0.0187	9	2.1 *	0.81	–0.223	0.0347	12	4.5 *
	Poultry	0.90	–0.506	0.0655	9	2.0	0.80	–0.492	0.0933	9	2.0 *
	WWTS	0.92	–0.214	0.0194	12	4.7 *,†	0.90	–0.391	0.0473	9	2.6 *,†

* There was a statistical difference between the T_{90} values storage and soil studies for a given bacteria, waste and temperature of incubation at a 5% level ($p < 0.05$). † There was a statistical difference between T_{90} values at different temperatures for a given bacteria and waste at a 5% level ($p < 0.05$).

T_{90} values obtained for *C. jejuni* were higher at 4 °C than at 14 °C and ranged from 4.8 to 9.8 days (bovine), 9.7 to 18.7 (pig), 19.0 to 21.7 (poultry), 21.1 to 26.8 (ovine), and 24.4 to 26.6 days (WWTS). Regardless of the temperature, *C. jejuni* was no longer viable after two months in bovine and pig and five months in poultry, ovine, and WWTS-amended soil. In the soil study, WWTS showed the highest T_{90} values ($p < 0.05$), followed by poultry and ovine, between which no significant differences were observed. Bovine and pig wastes demonstrated the lowest T_{90} values, also without significant differences between them.

The resistance of *L. monocytogenes* in soil showed a similar trend to the stored wastes and it was detected in all the samples at the end of the incubation period. The T_{90} values obtained at 4 and 14 °C were 2.0 and 2.0 (poultry), 2.1 and 4.5 (pig), 4.7 and 2.6 (WWTS), and 5.8 and 5.3 days (bovine), increasing up to 83.7 and 40.4 days in ovine-amended soil. The order of effect was ovine, bovine, pig, WWTS, and poultry; however, none of these differences were statistically significant.

C. sporogenes remained stable throughout the six months. Accordingly, the T_{90} values could not be calculated.

Overall, waste type had a statistically significant effect on all bacterial species examined. Temperature also showed a significant effect in all cases, with the exception of *L. monocytogenes* in the soil experiment ($p > 0.05$). A significant interaction between waste type and temperature was observed in all cases, except for *E. coli* O157:H7 during storage and *C. jejuni* and *L. monocytogenes* in amended soil ($p > 0.05$). The ranking of the effect of waste type on the pathogen survival is presented in Table 4.

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Table 4. Ranking of the different wastes according to their effect on pathogen survival.

Study	Pathogen	Temperature	Wastes Ranked by Their Effect on Survival
Storage	*S.* Typhimurium	4 °C	WWTS > Bovine > Poultry ≈ Pig > Ovine
		14 °C	WWTS > Bovine > Pig > Ovine ≈ Poultry
Soil	*S.* Typhimurium	4 °C	Ovine > WWTS > Bovine ≈ Pig > Poultry
		14 °C	WWTS > Poultry ≈ Ovine ≈ Bovine ≈ Pig
Storage	*E. coli* O157:H7	4 °C	Ovine ≈ Bovine > WWTS ≈ Pig ≈ Poultry
		14 °C	Ovine ≈ Bovine ≈ WWTS ≈ Pig ≈ Poultry
Soil	*E. coli* O157:H7	4 °C	Bovine > WWTS ≈ Ovine > Pig > Poultry
		14 °C	Ovine ≈ Bovine ≈ Pig ≈ WWTS ≈ Poultry
Storage	*C. jejuni*	4 °C	WWTS ≈ Ovine ≈ Poultry > Bovine ≈ Pig
		14 °C	WWTS > Ovine > Poultry > Bovine ≈ Pig
Soil	*C. jejuni*	4 °C	WWTS ≈ Ovine ≈ Poultry ≈ Pig > Bovine
		14 °C	Ovine ≈ WWTS ≈ Poultry ≈ Pig > Bovine
Storage	*L. monocytogenes*	4 °C	Pig > Bovine ≈ Ovine ≈ WWTS ≈ Poultry
		14 °C	Bovine > Ovine ≈ Pig ≈ WWTS ≈ Poultry
Soil	*L. monocytogenes*	4 °C	Ovine ≈ Bovine ≈ WWTS > Pig ≈ Poultry
		14 °C	Ovine ≈ Bovine ≈ Pig ≈ WWTS ≈ Poultry

Differences denoted by '>' are statistically significant ($p < 0.05$), whereas those indicated by '≈' are not statistically significant ($p > 0.05$).

A *t*-test was conducted to determine the difference between the T_{90} values obtained for a specific microorganism, type of waste, and temperature of incubation when the wastes are stored and when soil is amended with the wastes.

No significant differences ($p > 0.05$) in the survival of *S.* Typhimurium were observed when comparing waste and waste-amended soil across all samples and temperatures, with the exception of bovine waste at 14 °C, ovine waste at both temperatures, and wastewater at 4 °C.

For *E. coli* O157:H7, no significant differences ($p > 0.05$) in T_{90} values were found between stored waste and waste–amended soil, except for bovine waste and WWTS at 14 °C.

In the case of *C. jejuni*, the T_{90} values showed significant differences ($p < 0.05$) across all waste types and temperatures, except for ovine and poultry waste at 4 °C, and WWTS at 4 °C and 14 °C.

The T_{90} values for *L. monocytogenes* were significantly different ($p < 0.05$) across all samples and temperatures, except for soil amended with poultry waste at 4 °C and with ovine waste at 14 °C.

4. Discussion

In cattle slurry, the T_{90} values obtained for *S*. Typhimurium were 13.9 at 4 °C and 14.3 days at 14 °C. This is in agreement with Himathongkham (1999), who reported a T_{90} value for *S.* Typhimurium in cattle slurry of 16.4 days at 4 °C and 12.7 days at 20 °C [40]. Our study also achieved a 90% reduction in *S*. Typhimurium in swine slurry after 4.9 days at 4 °C and 2.4 days at 14 °C. Arrus et al. (2006) reported T_{90}-values ranged from 22 to 60 days at 4 °C and 8 to 19 days at 20 °C in manure from sow, feeder, and nursery barns [41]. A maximum survival of one month was achieved in the present study, in contrast to 84 days in pig manure obtained by McCarthy et al. (2015) when pig manure was inoculated with a cocktail of *S*. Typhimurium, *S*. Derby, and *S*. Infantis [42].

In soil amended with cattle manure, our T_{90}-values were 10.3 days at 4 °C and 5.4 days at 14 °C. Russel et al. (2022) reported T_{90}-values for *S*. Seftenberg of 16.7 days at 4 °C and 12.7 days at 14 °C [32]. Our data suggests maximum survival of three months at

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both temperatures, which constituted a longer survival than other studies, which reported 3 to 43 days at 4–5 °C [32,43], and from 6 to 28 days at 14–15 °C [23,32,43].

T_{90} values of 48.8 and 35.7 days were obtained for *E. coli* O157:H7 in cattle manure at 4 and 14 °C. Other studies have reported similar T_{90} values, ranging from 6.5 to 44.1 days at 4 °C and 18.1 to 33.3 days at 12.4 °C [40,44]. Regarding the maximum survival, the pathogen was still detected, albeit by enrichment, after six months of incubation in cattle slurry at both temperatures. Nicholson et al. (2005) reported a survival from 32 to up to 93 days [23]. A 90% reduction in O157 in pig slurry was obtained after 18.2 days, independent of the temperature. In accordance with our findings, Hutchinson et al. (2005) reported T_{90} values of 19.1 and 18.9 days at 4 °C and 20.0 and 25.2 days at 12.4 °C in manure from breeders and finishing pigs, respectively [44].

T_{90} values from the present study were 52.9 days at 4 °C for soil amended with bovine and 19.8 at 14 °C, respectively. Russell et al. (2022) reported that a 90% reduction in *E. coli* O157 was achieved when clay–loam soil was amended with bovine slurry (24.4 days at 4 °C and 25.8 at 14 °C) [32]. In our findings, *E. coli* O157 was still detectable after three months in soil amended with bovine and pig waste at 14 °C, four months in pig waste at 4 °C, and after more than six months in bovine waste at 4 °C. Regardless of incubation temperature and sample type, Nicholson et al. (2005) reported that the pathogen was detected up to 32 days in clay–loam soils amended with pig and bovine slurry [23].

C. jejuni was not detected immediately after the inoculation in bovine and pig waste in the present study. Hutchinson et al. (2005) investigated the survival of *C. jejuni* in cattle slurry and slurry derived from breeder and finishing pigs and obtained counts throughout their study. In cattle slurry, T_{90} values ranged from 8.2 to 15.5 days and 6.9 to 15.5 days at 12.4 °C. The corresponding values were 7.7 and 7.2 days at 4.3 °C and 14.4 and 11.4 days at 12.4 °C, for slurry from breeder and finishing pigs, respectively [44]. A maximum duration for survival of *C. jejuni*, up to 32 days, in bovine slurry has been described [23]. The pathogen survived up to 5 months in poultry litter. Previous studies have reported a maximum duration of *C. jejuni* of up to 5 days when poultry litter was stored at 20 °C [45,46].

The present study obtained T_{90} values for *L. monocytogenes* of 88.3 and 121.0 days in cattle slurry and 170.4 and 22.1 days in pig waste at 4 and 14 °C, respectively. In contrast, Hutchison et al. (2005) reported lower T_{90} values in dairy and beef cattle slurry from 9.4 to 16.0 days at 4.3 °C and 9.5 to 13.7 days at 12.4 °C, while the T_{90} values for breeder and finishing pig slurry were 14.1 and 16.1 days and 13.4 to 22.7 days at 4 and 14 °C, respectively [44].

Regarding the survival of *L. monocytogenes* in soil amended with bovine waste, T_{90} values of 83.7 and 40.4 days at 4 and 14 °C were obtained. Russell et al. (2022) reported T_{90} values of 12.8 days at 4 °C, and 6.0 days at 14 °C in bovine slurry applied to clay–loam soil [32]. In our findings, *L. monocytogenes* was still viable after six months of storage at both temperatures. Nicholson et al. (2005) reported a survival of 32 days when bovine slurry was applied to clay–loam soil [23].

The spore–forming bacteria *C. sporogenes* is ubiquitous in soil and faeces of both humans and animals. As a result of the resistance of spores to environmental stress, the concentration of these bacteria remained stable throughout the six months of storage, regardless of the incubation temperature. This is consistent with the results from Russell et al. (2022), who reported the persistence of these spores in inoculated cattle slurry and digestate when applied to clay–loam and sandy soil (five logs remained after 124 days) [32]. Girardin et al. (2005) had previously reported the maintenance of *C. sporogenes* in soil amended with composted wastewater treatment sludge and bovine slurry as a mere 0.7 log reduction after 16 months [31].

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Regardless of the sample type, bacterial survival has been reported to be greater at lower temperatures due to reduced biological activity. Higher temperatures accelerate the die-off because of microbial competition and the decomposition of organic compounds into ammonia and VFAs, which are bactericidal [30,47]. In the current study, there was no clear effect of temperature on survival. Of the forty combinations, the T_{90} values were significantly higher at 4 C in nine scenarios and significantly lower in three.

The overall pH increased regardless of the sample type and temperature of incubation or treatment and ranged at the end of the experiment from neutral to slightly alkaline (7.4–9.4). This might have been expected to facilitate survival but, according to Ravva and Sarreal (2014), the destruction of *Salmonella* in poultry may be connected to the increase in pH and consecutive release of ammonia [48].

We initially hypothesised that the survival of pathogens would be accelerated during the application of wastes into soil, due to soil microbiota competition. However, of the forty different combinations of bacteria, type of waste, and temperature of incubation, there was a significant difference between the two treatments (waste versus waste plus soil) in 20 of them, specifically in *L. monocytogenes* and *C. jejuni.* T_{90} values from storage were significantly higher 11 times and lower 9 times. Higher survival rates have been previously reported in manure-amended soil compared to cattle manure alone. In particular, the maximum survival of two strains of *S.* Newport more than doubled from 49 days in manure to 107 days in cattle manure-amended soil. The authors hypothesised that this could be related to a steady pH increase from 6.70 on day 0 to 8.00 on day 72 [49], although pH values for the amended soil were not reported. In the present study, the pH increase was more pronounced in the stored-waste-only samples compared to the amended soil, suggesting that pH alone does not account for the greater pathogen survival observed in soil.

5. Conclusions

In conclusion, *S.* Typhimurium was eliminated after three months of storage regardless of waste type or the addition to soil, while a similar result was obtained with *C. jejuni* after five months. However, *E. coli* O157:H7, *L. monocytogenes*, and *C. sporogenes* were still detected after six months. These results suggest that there are no safe storage temperatures and/or time allowances to assure pathogen destruction. Consequently, the application of manure or biosolids to land can potentially lead to soil and water contamination, potentially facilitating the transmission of pathogens to animal and humans. Specific interventions are therefore required to prevent the cross-infection of animals and cross-contamination of crops and the environment, in order to protect the environment and animal and human health.

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Abbreviations

The following abbreviations are used in this manuscript:

GVA	Gross Value Added
EC	European Commission
GHG	Greenhouse Gas
CFU	Colony-forming units
VFA	Volatile Fatty Acids
ARG	Antimicrobial resistant genes
AMR	Antimicrobial resistance
WHO	World Health Organisation
STEC	Shiga toxin-producing *Escherichia coli*
FBO	Foodborne Outbreaks
WWTS	Wastewater treatment sludge
VBNC	Viable but not culturable
DM	Dry matter

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Biostimulatory Effects of *Bacillus subtilis* and *Pseudomonas corrugata* on Phytochemical and Antioxidant Properties of In Vitro-Propagated Plants of *Nardostachys jatamansi* (D. Don) DC

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Abstract: Plant growth-promoting rhizobacteria (PGPRs) are well known for their capacity to enhance the growth and survival of in vitro-grown plants. However, their effect on *Nardostachys jatamansi* (D. Don) DC., a critically endangered medicinal plant in the Indian Himalayan Region, is still unknown. In this study, a simple, reproducible protocol for in vitro propagation of *N. jatamansi* was established using shoot tip explants, cultured on Murashige and Skoog (MS) medium supplemented with different plant growth regulators, including N6-benzylaminopurine, thidiazuron (TDZ), and naphthalene acetic acid (NAA). MS media supplemented with 2.0 µM TDZ and 0.5 µM NAA created a significant shoot induction with an average of 6.2 shoots per explant. These aseptically excised individual shoots produced roots on MS medium supplemented with Indole Butyric Acid or NAA within 14 days of the transfer. The PGPR, viz., *Bacillus subtilis* and *Pseudomonas corrugata*, inoculation resulted in improved growth, higher chlorophyll content, and survival of in vitro-rooted plants (94.6%) after transfer to the soil. Moreover, the PGPRs depicted a two-fold higher total phenolics (45.87 mg GAE/g DW) in plants. These results clearly demonstrate the beneficial effects of *P. corrugata* and *B. subtilis* on the growth, survival, and phytochemical content of *N. jatamansi*.

Keywords: antioxidant activity; *Nardostachys jatamansi*; PGPRs; Indian Himalayan region

1. Introduction

Nardostachys jatamansi (D.Don) DC., locally known as Jatamansi, is a small, erect, hairy, perennial rhizomatous herbaceous plant belonging to the Valerianaceae family. It is distributed in the steep and open areas of the Himalayan regions of India, Nepal, and Bhutan between 3000 and 5200 above mean sea level (a.m.s.l) [1]. The most useful portion of the plant, the rhizomes, is dark brown, aromatic, tapering, and heavily covered in reddish-brown fibrous remnants of old leaf bases. This gives the rhizomes the appearance of a thick beard, hence the name "jatamansi" [2].

For many years, the traditional uses of *N. jatamansi* have included the stimulant, hepatoprotective, cardioprotective, sedative, neuroprotective, and antidiabetic properties of both its roots and rhizomes. The roots are also used to treat headaches, epilepsy, menopausal symptoms, intestinal colic, and flatulence [3–5]. According to phytochemical studies, *N. jatamansi* is a valuable source of essential oils that are rich in coumarins and sesquiterpenes. It also contains seselin, nardol, jatamansinone, valeranone, jatamansinol, nardosinone, jatamansic acid, nardal, nardin, oroselol, jatamansin, spirojatamol, and dihydropyranocoumarin [6].

Pharmaceutical industries mostly obtain this important plant species directly from their natural habitat in order to meet their demands. The species of *N. jatamansi* is becoming rare within a few habitats and has now been included in the list of Himalayan Critically Endangered (CR) plants because of the depletion of naturally growing populations caused by uncontrolled collection, unsustainable grazing, and rising raw material demand. Furthermore, according to Mulliken and Crofton (2008), *N. jatamansi* is among the (top seven Asian) CITES-listed plants, and as such, careful harvesting and conservation are necessary [7].

The most common methods for propagating *N. jatamansi* are seeds and vegetative parts. However, only 10–20% of seeds germinate under natural conditions, which is very low and is one of the primary factors in the extinction of this important species [8]. A brief reproductive phase follows a lengthy 3–4-year juvenile phase in plants. As a result, the in vitro approach can greatly aid in the mass reproduction and preservation of this extremely endangered species. Although there have been a few reports on the in vitro propagation of *N. jatamansi* [2,9–13], the effects of PGPR on phytochemical parameters, growth, and survival have not yet been investigated.

The transfer of in vitro-propagated plants during acclimatization and their survival is the most crucial step in the plant tissue culture technique. During the hardening and acclimatization of in vitro-grown plantlets, most plants are unable to tolerate the attack of soil microbes and environmental conditions. Because of this microbial attack and environmental change, 10–40% of in vitro-regenerated juvenile plants either die or are unable to compete in the market, which causes a significant loss to investors at the commercial level [14,15]. To overcome this problem, several researchers have reported the use of PGPRs during the hardening of in vitro-propagated plants, which is also known as biological hardening [16–22]. The effectiveness of such plant growth-promoting rhizobacteria (PGPRs) in biological hardening to enhance the growth, survival, and secondary metabolites of tissue culture-raised plants has been realized and reported by several researchers [16,17,23–27]. Many soil microorganisms like *Bacillus*, *Pseudomonas*, *Azospirillum*, etc., have been proposed as a suitable PGPR for agricultural crops, considering their phytobeneficial and abiotic stress tolerance properties [28–32]. In particular, the application of *Bacillus subtilis* and *Pseudomonas corrugata* has been explored for improving the acclimatization and adaptation of in vitro-regenerated plants by promoting root development, enhancing nutrient uptake, and inducing systemic resistance, thereby facilitating a more successful transition to ex vitro conditions [32–34].

Several studies have highlighted the plant growth-promoting potential of *Bacillus subtilis* and *Pseudomonas corrugata*, particularly in enhancing the adaptation of in vitro-propagated plants [34]. These rhizobacteria exert a wide range of PGPR activities, including phosphate solubilization, nitrogen fixation, production of indole-3-acetic acid (IAA), siderophore production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, which collectively contribute to improved plant growth and stress tolerance [35–37]. *B. subtilis* has been widely reported for its ability to enhance root architecture and nutrient

uptake through IAA production and phosphate solubilization [38,39]. Similarly, *P. corrugata* exhibits strong siderophore production and ACC-deaminase activity, which help alleviate ethylene-induced stress during plant acclimatization [35,40]. The synergistic interaction of these traits aids in successful ex vitro establishment and overall growth promotion of tissue culture-derived plants, thereby supporting their use in biological hardening protocols.

Based on the above-mentioned points, the current study aimed to (1) develop an efficient in vitro propagation protocol of *N. jatamansi* from the shoot tip; (2) evaluate the effect of PGPRs, viz., *Bacillus subtilis* and *Pseudomonas corrugata*, on the growth, phytochemical parameters, and antioxidant activities of in vitro-propagated plants; and (3) analyze genetic fidelity using ISSR markers.

2. Materials and Methods

2.1. Plant Material and Explant Selection

In vitro propagation studies of *N. jatamansi* were carried out in the laboratory on matured seeds collected during October from the Tungnath and Chopata region of the Rudraprayag district of Uttarakhand, India, at altitudes ranging from 30°29′21.79″ N to 79°13′0.23″ E 3444 m amsl.

To obtain in vitro plants, mature seeds of *N. jatamansi* were surface sterilized using a 70% ethanol (*v*/*v*) solution for one minute, followed by treatment with a 0.2% mercuric chloride solution for 5 min. The treated seeds were then thoroughly rinsed with sterile water five times and left to soak in sterile distilled water for 24 h in the dark to soften the seed coat. These hydrated seeds were then placed onto Murashige and Skoog basal medium containing 0.8% *w*/*v* agar at pH 5.8, with one seed per tube [41].

Test tubes containing shoot tips (5–10 mm) with two leaf primordia from 15-day-old in vitro seedlings were transferred to the culture room. The culture room had a light intensity of 100 $\mu Em^{-2}\ s^{-1}$, a photoperiod of 16 h per day, and a relative humidity of 70%. The explants were then used for shoot multiplication.

2.2. Induction of Shoot Buds

Multiple combinations of plant growth regulators, that is, benzylaminopurine (BAP), Indole-3-acetic acid (IAA), and thidiazuron (TDZ), at varying concentrations were tested on Basal MS medium for their ability to induce multiple shoot formation (Table 1). Each trial was replicated twice using ten explants per treatment to ensure the accuracy and reliability of the data obtained.

Table 1. Effect of different concentrations of plant growth regulators on in vitro regeneration from rhizome explants of *N. jatamansi* after 28 days of culture. Values represent mean ± SE of 10 replicates/treatment, and all the experiments were repeated twice. Means followed by the same letter in the column are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Plant Growth Regulators (μM)			No. of Shoots	Shoot Length (cm)	No. of Leaves
BAP	TDZ	NAA			
0.0	0.0	0.0	1.6 ± 0.2^{j}	2.8 ± 0.1^{f}	1.6 ± 0.2^{i}
1.0	0.0	0.1	2.1 ± 0.2^{i}	3.3 ± 0.2^{e}	1.8 ± 0.3^{h}
2.5	0.0	0.1	2.3 ± 0.2^{h}	3.6 ± 0.2^{d}	2.4 ± 0.2^{g}
5.0	0.0	0.1	2.1 ± 0.2^{i}	2.7 ± 0.3^{g}	2.3 ± 0.4^{g}
1.0	0.0	0.5	1.2 ± 0.6^{k}	4.1 ± 0.6^{ab}	3.2 ± 0.3^{e}
2.5	0.0	0.5	3.3 ± 0.3^{f}	3.7 ± 0.5^{c}	3.2 ± 0.7^{e}
5.0	0.0	0.5	3.8 ± 0.2^{e}	2.9 ± 0.2^{f}	2.8 ± 0.4^{f}

Table 1. *Cont.*

Plant Growth Regulators (μM)			No. of Shoots	Shoot Length (cm)	No. of Leaves
BAP	TDZ	NAA			
0.0	1.0	0.1	2.8 ± 0.4^{g}	4.0 ± 0.7^{ab}	3.3 ± 0.3^{e}
0.0	2.0	0.1	4.5 ± 0.3^{c}	3.8 ± 0.6^{bc}	3.8 ± 0.5^{d}
0.0	4.0	0.1	5.8 ± 0.4^{b}	4.1 ± 0.4^{ab}	4.4 ± 0.4^{a}
0.0	1.0	0.5	4.2 ± 0.2^{d}	4.1 ± 0.5^{ab}	4.2 ± 0.6^{b}
0.0	2.0	0.5	6.2 ± 0.3^{a}	4.2 ± 0.4^{a}	4.1 ± 0.5^{bc}
0.0	4.0	0.5	4.2 ± 0.2^{d}	3.9 ± 0.4^{b}	4.1 ± 0.4^{bc}

2.3. Rhizogenesis

Proliferating shoots, measuring approximately 3 to 4 cm in length, were inoculated on Murashige and Skoog (MS) medium supplement with different concentrations of IAA at a concentration range of 0.0–1.0 μM and Naphthaleneacetic acid (NAA) at a concentration range of 0.0–0.5 μM, either separately or in combination, for rooting (see Table 2).

Table 2. Effect of auxin supplementation on rooting response of *N. jatamansi.* Values represent mean ± SE of 10 replicates/treatment, and all the experiments were repeated twice. Means followed by the same letter in the column are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Growth Regulator + 1/2 MS		Shoot Forming Roots (%)	Number of Days to Root	Number of Roots/Shoots	Root Length (cm)
IAA (μM)	NAA (μM)				
0.0	0.0	21.3 ± 0.8^{j}	14–21	1.1 ± 0.2^{i}	2.8 ± 0.4^{e}
0.1	0.0	53.6 ± 0.8^{f}	15–20	2.3 ± 0.3^{g}	2.9 ± 0.3^{d}
0.5	0.0	89.6 ± 1.2^{a}	15–20	3.6 ± 0.7^{a}	3.4 ± 0.4^{b}
0.0	0.1	51.2 ± 0.7^{g}	10–21	2.1 ± 0.4^{h}	3.6 ± 0.5^{a}
0.1	0.1	66.6 ± 1.0^{d}	10–21	2.2 ± 0.6^{gh}	2.8 ± 0.4^{e}
0.5	0.1	64.6 ± 1.3^{e}	18–24	3.4 ± 0.4^{b}	2.6 ± 0.6^{f}
0.0	0.5	73.4 ± 0.7^{c}	10–21	2.1 ± 0.2^{h}	3.1 ± 0.4^{cd}
0.1	0.5	42.3 ± 1.1^{i}	12–18	3.1 ± 0.6^{cd}	3.4 ± 0.6^{b}
0.5	0.5	66.6 ± 1.1^{d}	12–18	2.9 ± 0.2^{e}	3.2 ± 0.6^{c}
0.0	1.0	73.7 ± 1.4^{c}	14–21	3.2 ± 0.8^{c}	3.6 ± 0.5^{a}
0.1	1.0	48.3 ± 0.7^{h}	10–21	3.4 ± 0.6^{b}	3.4 ± 0.9^{b}
0.5	1.0	78.2 ± 0.9^{b}	12–18	2.6 ± 0.6^{f}	3.2 ± 0.8^{c}

2.4. Hardening of Plantlets and Inoculation Using PGPRs

N. jatamansi plants, which were raised using tissue culture, were transferred to plastic trays containing a mixture of sand, soil, and compost at a 1:1:1 ratio. To ensure proper humidity, the plants were initially covered with transparent polythene. After the second week, the covers were temporarily removed for 2–3 h each day to facilitate acclimatization. By the fourth week, the cover had been completely withdrawn.

The experimental inoculation involved the utilization of two bacterial strains: *Bacillus subtilis* (MTCC 8528) and *Pseudomonas corrugata* (ATCC 29736) [16,34]. Both the isolates were taken from the institute's laboratory. In the present study, the inclusion of these two bacterial strains was based on their proven roles in promoting plant growth through physiological and biochemical mechanisms relevant to the goals of in vitro plant acclimatization. These bacteria have been widely reported to enhance plant development and

stress resilience through various biochemical mechanisms. Specifically, *B. subtilis* strains are known to solubilize phosphate, fix atmospheric nitrogen, produce indole-3-acetic acid (IAA), and secrete siderophores and ACC-deaminase, all of which collectively enhance nutrient uptake, root architecture, and tolerance to environmental stresses [42–44]. Similarly, *P. corrugata* has been shown to possess high ACC-deaminase activity, siderophore production, and IAA biosynthesis, contributing significantly to plant vigor and successful adaptation, particularly under in vitro to ex vitro transition phases [16,45,46].

The inoculation process was conducted on four-week-old *N. jatamansi* plantlets by adding a broth solution containing 10^6 colony-forming units (cfu)/mL to the planting hole prior to planting, according to the designated treatment. 1 mL of a 10^6 CFU/mL suspension per gram of soil. This ensures a consistent and measurable concentration of microbes in the soil sample [47].

Both in vitro- and seed-grown plants were included in the analysis. Each treatment consisted of twenty-five plants grown under regular soil conditions without plant growth-promoting rhizobacteria (PGPR), which served as a control.

2.5. Data Recording

After 60 d of inoculation, plant growth was assessed in terms of plant height, leaf area, number of leaves, root length, and fresh and dry weights of shoots and roots. In addition, the chlorophyll content of the leaves was measured using the Arnon method [30] with 80% acetone extract. To determine any significant differences between the mean values, statistical analysis was conducted using Duncan's multiple range test (DMRT) with Statistical Software v5.0 (StatSoft 1995) [48], with a significance level set at $p \leq 0.05$.

2.6. Preparation of Sample Extract

The sample for analysis was prepared by mixing 1 g of the powdered sample of dried roots with 5 mL of 80% methanol, followed by thorough grinding in a mortar and pestle. The resulting homogenate was then centrifuged at 8000 rpm for 15 min, and the supernatant was carefully collected. The residue was washed with 50 mL of 80% methanol. The combined filtrate (methanol) was stored at 4 °C until required. This methanolic extract was used to analyze the levels of total phenols, alkaloids, flavonoids, tannins, and antioxidant activity.

2.7. Analysis of Total Phenol, Total Alkaloids, Tannin, and Flavonoids

Total phenolic and tannin contents were quantified according to the method outlined by Swain and Hillis [49]. The total alkaloid content was determined following the protocol established by Sreevidya and Mehrotra [50], whereas flavonoids were assessed using the method described by Kim et al. [51]. The total flavonoid, phenolic, tannin, and alkaloid content was expressed as milligrams of per gram of dry weight, milligrams of gallic acid equivalent (GAE) per gram of dry weight sample, milligrams of tannic acid quercetin equivalent (QE)equivalent (TAE) per gram of dry weight sample, and milligrams of atropine equivalent (AE) per gram of dry weight sample, respectively.

2.7.1. Antioxidant Activity

Two different in vitro assays, i.e., 2,2-diphenyl-1-picryylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, were used to estimate the antioxidant activity of each sample.

2.7.2. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The effect of the methanolic extract of *N. jatamansi* on DPPH radicals was measured using a previously described method Rawat et al. [52]. The absorbance of the sample was recorded against a reference (control; solution of methanol and DPPH). The DPPH radical scavenging activity of the species was estimated at concentrations ranging from 250 μg/mL to 1000 μg/mL. The antioxidant activity results are presented in mM ascorbic acid equivalent (AAE) per g dry weight of the sample. The following formula was used to calculate the scavenging capacity of the samples:

Scavenging (%) = (absorbance of the control at 517 nm − absorbance of the sample) × 100% absorbance of the control

2.7.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing ability of the methanolic extract was determined using the method of [53]. The absorbance of the solution (methanolic extract and FRAP reagent) was measured spectrophotometrically at 593 nm wavelength. Ascorbic acid (10–100 μg/mL) was used as a standard to determine the FRAP value, and the results were presented in mM ascorbic acid equivalent (AAE) per gram dry weight of the sample.

3. Results

3.1. Seed Germination, Shoot Induction, and Multiplication

Multiple combinations of plant growth regulators have been used to develop effective and efficient in vitro micropropagation protocols for *N. jatamansi*. Shoots were regenerated using shoot-tip explants from in vitro seedlings. Within one week of culture, shoot buds were induced on basal medium containing 3% sucrose (Figure 1a,b); however, shoot growth was suppressed in the absence of any plant growth regulators. Several combinations of plant growth regulators, such as IAA (0.0–0.5 μM), BAP (0.0–5.0 μM), and TDZ (0.0–4.0 μM), were used to promote shoot multiplication and elongation (Table 1). The results, recorded after 2 weeks, were further analyzed and are presented in Table 1. MS medium supplemented with 0.5 μM NAA and 2.0 μM TDZ produced the highest (6.2 shoots per explant; Figure 1c) shoot proliferation in terms of induction and elongation within 4 weeks. According to Bose et al. [9], the addition of 1.0 mg/L of meta-Topolin in the medium is effective in inducing numerous shoots, but the findings of the current study indicated that the best shoot production was achieved by combining a low concentration of NAA with high TDZ. These results substantiate previous findings [14,36,37], which demonstrated that the combination of auxin and cytokinin positively influences growth in the culture medium. The media supplied with 0.5 μM NAA and 2.0 μM TDZ was also found best for maximum shoot length; however, the medium supplemented with 4.0 μM TDZ and 0.1 μM NAA exhibited the maximum number of leaves. Earlier, Rawat et al. [53] reported the shoot proliferation (4.5 shoots) in MS media supplemented with 5.0 μM BAP; however, in this study, the use of NAA and TDZ in combination gives 6.2 shoots. These results clearly show that the auxin/cytokinin ratio in the culture medium plays an important role in the morphogenetic response of cultured tissues.

3.2. Rhizogenesis of In Vitro-Developed Shoots

To induce rhizogenesis in the regenerated shoots, 2–4 cm elongated shoots were subcultured on basal MS medium, with or without the addition of plant growth regulators. A total of 12 media combinations containing IAA or NAA (individually or in combination) were used, which helped in root induction in plantlets. The concentrations of both plant growth regulators played a crucial role in influencing the percentage of shoot-forming roots and the number of roots per shoot, as outlined in Table 2. The number of shoots displaying

root initiation, as well as the number of roots induced per shoot, were documented after four weeks of cultivation.

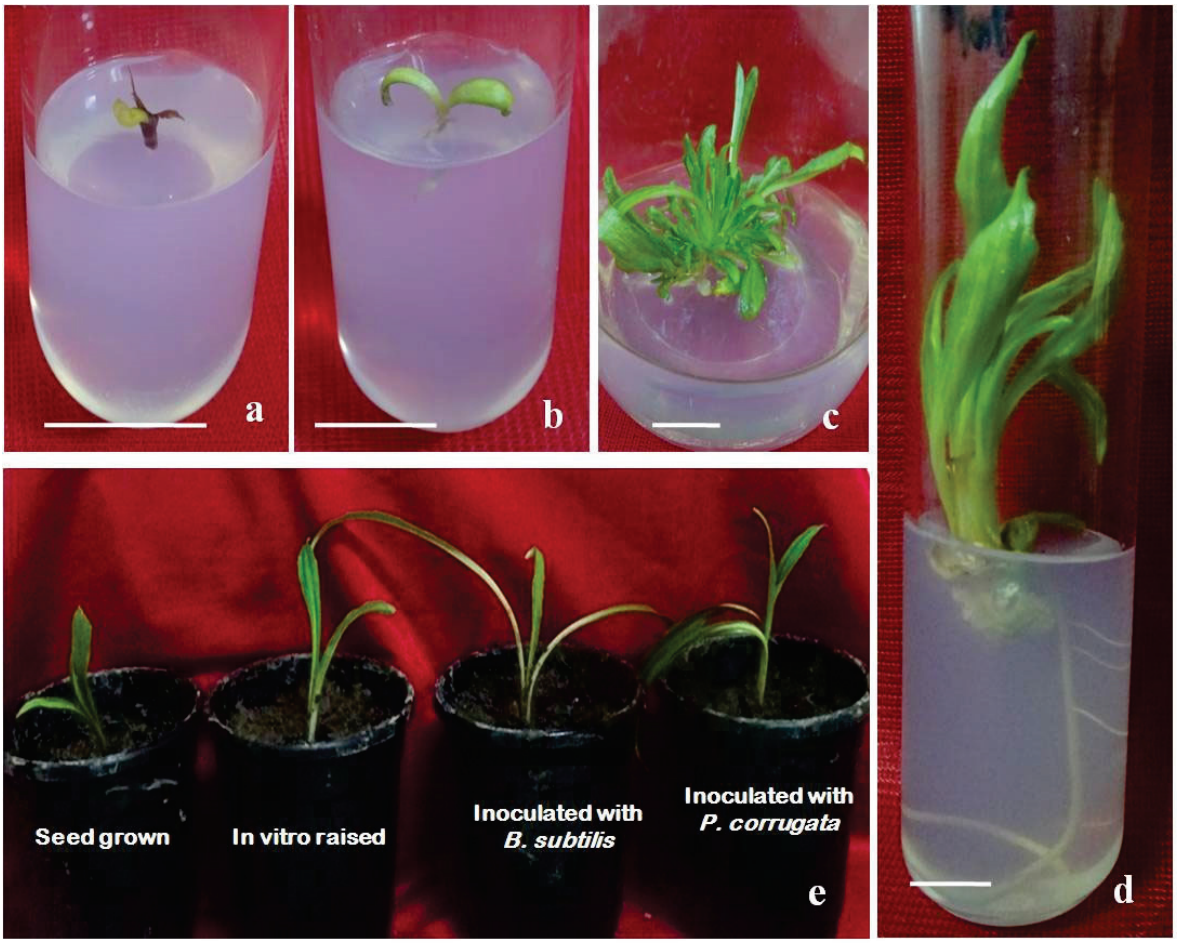

Figure 1. In vitro propagation of *Nardostachys jatamansi* (**a**,**b**) seed germination on basal MS medium. (**c**) Elongation and multiplication of shoots on MS media supplemented with 2.0 µM TDZ and 0.5 µM NAA. (**d**) Rooting in MS medium supplemented with 0.5 µM IAA. (**e**) Comparison of inoculated and uninoculated plants after 60 days of inoculation; bar = 1 cm.

Comparatively, IAA showed a better response than NAA in terms of the mean number of roots per shoot and percentage of shoot-forming roots. In contrast, the medium supplemented with 1.0 µM NAA showed maximum root length. High frequency of rhizogenesis (89.6%) in in vitro shoots was observed on media having 0.5 µM IAA (Figure 1d). The results of the current study, which showed that the presence of individual auxins (IAA or IBA) reduced the number of shoot-producing roots, are also supported by previous studies on *Aconitum balforii* [54,55] and *Digitalis ferruginea* [14].

3.3. Acclimatization with PGPR and Field Establishment

The results showed that 78.6% of the plants survived after 4 weeks of transfer to pots filled with soil, sand, and compost (1:1:1: *v*/*v*) and maintained in F conditions (controlled environment structure where temperature 25–30 °C, humidity 60–65%, and light are regulated to support the hardening and acclimatization of in vitro-cultured plants before transferring them to open fields). These tissue-cultured raised plants were utilized after four weeks to study the impact of PGPRs on growth and phytochemical parameters.

Inoculation of micropropagated plants of *N. jatamansi* with the two bacterial isolates was effective in enhancing the survival rate of plants after transfer to the soil (Figure 2, Table 3). The plants inoculated with *P. corrugata* showed the maximum survival rate (94.6%) after 60 d of inoculation. The survival rate was 85.3% in the plants inoculated with *B. subtilis*, whereas only 66.3% survival was observed in tissue culture-raised plants. In the control, only 18.4% of the seeds germinated and survived under natural conditions. After 60 d of transfer, the plants grew well and attained 8–10 cm in height. The acclimatized plants showed normal growth without phenotypic variation.

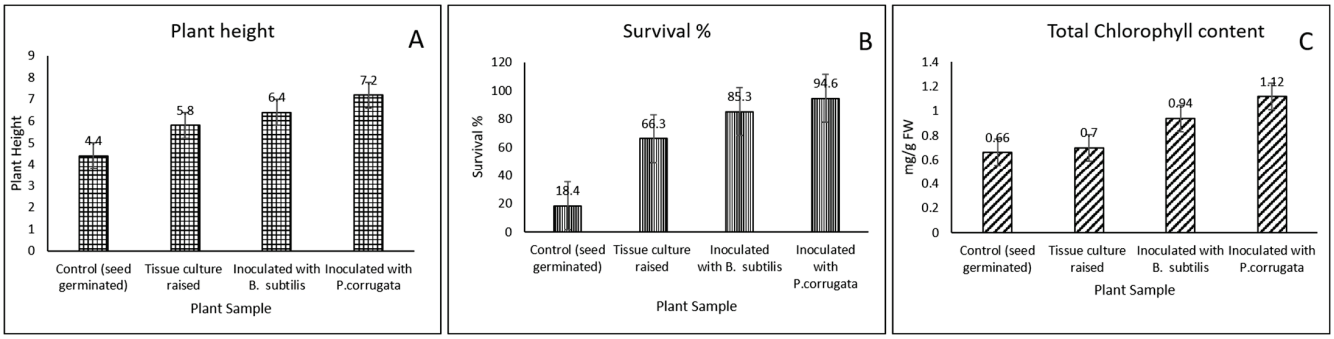

Figure 2. Impact of PGPR on plant height (**A**), survival percent (**B**), and chlorophyll content (**C**) of *N. jatamansi* after 60 days of inoculation with *B. sabtilis* and *P. corrugata*.

Table 3. Effect of PGPR on growth, survival, and chlorophyll content of in vitro-raised plants and inoculated plants of *N. jatamansi* after 60 days of inoculation. Values represent mean ± SE of 25 replicates/treatment. Means followed by the same letter in the column are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Plant Sample	Growth Parameters									
	Root Length	Root		Shoot		Leaf Area	No. of Leaves	Plant Height	Survival %	Chlorophyll Content
		FW	DW	FW	DW					
Control (seed germinated)	3.5 ± 0.2^{c}	0.83 ± 0.02^{d}	0.11 ± 0.01^{d}	1.62 ± 0.2^{c}	0.43 ± 0.1^{d}	2.42 ± 0.1^{d}	2.3 ± 0.2^{d}	4.4 ± 0.6^{d}	18.4 ± 1.2^{d}	0.66 ± 0.1^{c}
Tissue culture-raised	3.6 ± 0.5^{c}	1.02 ± 0.2^{c}	0.23 ± 0.01^{c}	2.42 ± 0.4^{b}	0.84 ± 0.1^{c}	3.65 ± 0.2^{c}	3.2 ± 0.6^{c}	5.8 ± 0.6^{c}	66.3 ± 2.2^{c}	0.70 ± 0.2^{c}
Inoculated with *B. subtilis*	3.9 ± 0.6^{b}	1.15 ± 0.4^{b}	0.31 ± 0.01^{b}	2.64 ± 0.3^{b}	0.96 ± 0.1^{b}	4.45 ± 0.7^{b}	3.8 ± 0.8^{b}	6.4 ± 0.8^{b}	85.3 ± 3.6^{b}	0.94 ± 0.7^{b}
Inoculated with *P.corrugata*	4.8 ± 0.4^{a}	1.43 ± 0.3^{a}	0.36 ± 0.01^{a}	3.22 ± 0.7^{a}	1.16 ± 0.2^{a}	4.83 ± 0.7^{a}	4.6 ± 0.6^{a}	7.2 ± 0.8^{a}	94.6 ± 4.3^{a}	1.12 ± 0.6^{a}

3.4. Effect of PGPR on Growth Parameters

Bacterial inoculation had a positive impact on various aspects of plant growth parameters, such as plant height, leaf area, number of leaves, root length, and fresh and dry weights of shoots and roots. Among the various treatments, the *P. corrugata*-inoculated plants showed the maximum increase in growth parameters compared to the *B. subtilis*-inoculated and -uninoculated plants (Table 1; Figure 1e). Singh et al. also reported an increase in aboveground growth in *Coleus forskohlii* plants colonized by *Pseudomonas monteilii* and *Glomus fasciculatum* [56]. The study discovered that these plants had greater aboveground biomass and higher rates of N, P, and K absorption. In another study, field tests were used by Aseri and coworkers to assess the effectiveness of PGPR, such as *A. chroococcum* and *A. brasilense*, and AM fungi, such as *G. mosseae* and *G. fasciculatum*, on the development, uptake of nutrients, and biomass production of pomegranate (*Punica granatum* L.) [40,57].

The chlorophyll content in the *N. jatamansi* plants was significantly increased by approximately 40% with PGPR inoculation compared to the uninoculated plants. The chlorophyll content was 1.12 mg/g FW and 0.94 mg/g FW in the plants inoculated with *P. corrugata* and *B. subtilis*, respectively, and 0.70 mg/g FW in the uninoculated plants. Thus, the statement indicates that an increase in chlorophyll content results in a higher photosynthetic rate, as well as improves plant biomass. A similar effect of PGPR inoculation on the chlorophyll content of *Punica grantum* after four months was also noted by Aseri et al. [40]. Additionally, the efficiency of plants in terms of root and shoot biomass, as well as the chlorophyll and NPK content, was significantly increased by inoculation with a single microbe [24,58,59].

3.5. Effect of PGPR on Total Phenol, Total Alkaloids, Tannin, and Flavonoids

Plant secondary metabolites are valuable resources for fragrances, food additives, flavors, pharmaceuticals, and various other industrially significant compounds. These metabolites play a crucial role in protecting plants from various threats, including herbivores, phytopathogens, and insect pests, as well as in helping plants withstand biotic and abiotic stresses. Environmental stressors, whether biotic or abiotic, increase the synthesis rate of secondary metabolites. These stressors can be microbial, physical, or chemical in origin [60–62]. Rhizosphere microorganisms, including PGPRs, are biotic elicitors that can induce the production of secondary metabolites [32,63–65].

The impact of PGPR strains *P. corrugata* and *B. subtilis* on total phenolic, total alkaloid, tannin, and flavonoid content was examined in in vitro-propagated plants of *N. jatamansi*. The plants inoculated with *P. corrugata* exhibited significantly higher levels of total phenolics, total alkaloids, tannins, and flavonoids than the uninoculated plants of *N. jatamansi*. Specifically, the total phenolic content was approximately two-fold higher in the *P. corrugata*-inoculated plants (45.87 mg GAE/g DW) than in uninoculated control plants (23.87 mg GAE/g DW). *B. subtilis* also displayed a stimulatory effect on the phytochemical parameters, but the values were lower than *P. corrugata*. The lesser effect of *B. subtilis* strain was attributed to its poor adaptation to root exudates and/or insufficient root colonization. Both *P. corrugata* and *B. subtilis* showed almost similar outcomes (33.89 mg AE/ g DW and 33.37 mg AE/ g DW, respectively) in the case of total alkaloid production. The increase in total phenolic, alkaloid, tannin, and flavonoid contents in response to PGPR inoculation was due to the increase in plant dry weight and growth-promoting chemicals synthesized by microbes. These substances influence plant metabolic pathways [32,63–65]. Palermo et al. reported a significant increase in total phenolic content exclusively in plants that were both inoculated with *Bacillus velezensis* strain GB03 and subjected to larval damage [49]. The study also highlighted the complex interactions among beneficial microbes, herbivores, and plant defense responses, underscoring their potential role in enhancing plant resilience and boosting the production of secondary metabolites [63–65]. PGPR inoculation markedly improved the growth and nutrient uptake of *Cucumis sativus* seedlings, especially under higher substrate moisture levels [66]. Significant increases were observed in plant height, root length, and fresh weight, with synergistic effects from PGPR and moisture resulting in up to 197% and 267% increases in height and root length, respectively [66]. Similar observations have been documented in previous studies on *Hyoscyamus niger* [60], *Stevia rebaudiana* [24], and *Salvia officinalis* [67]. The plants grown from the seeds displayed minimum values for all four parameters (Figure 3, Table 4).

Table 4. Comparison of total phenol, alkaloids, tannin, flavonoids, and antioxidant activity of the in vitro-raised plants and inoculated plants of *N. jatamansi*. Values represent mean ± SE of 25 replicates/treatment. Means followed by the same letter in the column are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Plant Sample	Total Phenol (mg GAE/g DW)	Alkaloids (mg AE/g DW)	Tannin (mg TAE/g DW)	Flavonoids (mg QE/g DW)	Antioxidant Activity (mM AAE/g DW)	
					DPPH	FRAP
Control (seed germinated)	23.87 ± 2.18 ^{d}	21.87 ± 3.12 ^{c}	11.71 ± 1.13 ^{d}	24.12 ± 2.63 ^{d}	43.13 ± 4.8 ^{d}	2.11 ± 0.3 ^{d}
Tissue culture-raised	29.87 ± 2.18 ^{c}	25.81 ± 3.88 ^{b}	16.33 ± 2.23 ^{c}	29.33 ± 3.13 ^{c}	55.88 ± 5.1 ^{c}	3.88 ± 0.9 ^{c}

Table 4. *Cont.*

Plant Sample	Total Phenol (mg GAE/g DW)	Alkaloids (mg AE/g DW)	Tannin (mg TAE/g DW)	Flavonoids (mg QE/g DW)	Antioxidant Activity (mM AAE/g DW)	
					DPPH	FRAP
Inoculated with *B. subtilis*	31.36 ± 2.45 ^{b}	33.37 ± 3.44 ^{a}	21.76 ± 2.66 ^{b}	32.66 ± 3.42 ^{b}	63.46 ± 5.3 ^{b}	5.44 ± 0.9 ^{b}
Inoculated with *P.corrugata*	45.87 ± 3.18 ^{a}	33.89 ± 3.98 ^{a}	28.39 ± 3.12 ^{a}	39.67 ± 3.54 ^{a}	67.89 ± 5.3 ^{a}	6.76 ± 1.0 ^{a}

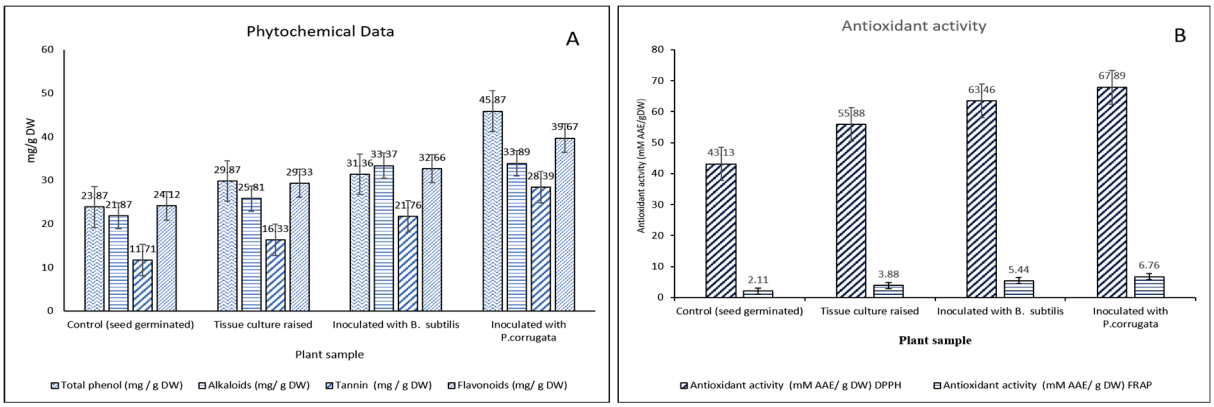

Figure 3. Graphical representation of total phenol, alkaloids, tannin, flavonoids (**A**), and antioxidant activity (**B**) of control, in vitro-raised plants, and PGPR-inoculated plants of *N. jatamansi*.

3.6. Antioxidant Activity

Plants have different types of secondary metabolites such as tannins, phenolics, flavonoids, and alkaloids. These compounds are known to act as scavengers of free radicals and are anti-carcinogenic, anti-inflammatory, and antimutagenic [68–73], and others have reported that the powerful antioxidant activity of these phytochemicals improves human health by modulating metabolism. Furthermore, it is also useful in treating diseases that are caused by deregulation of free radical generation in cells, such as cardiovascular diseases, cancer, inflammatory disorders, and diabetes.

The total antioxidant activity, measured by DPPH and FRAP, varied significantly ($p < 0.05$) between the inoculated and uninoculated plants (Table 4). According to the reducing ability, both assays revealed that the inoculated plants exhibited higher antioxidant activity than the control plants (Table 4). The plants inoculated with *P. corrugata* and *B. subtilis* showed 6.76 and 5.44 mM AAE/g DW antioxidant activities, respectively, in the FRAP assay. In the DPPH assay, the antioxidant activities were 67.89 and 63.46 mM AAE/g DW for the *P. corrugata*- and *B. subtilis*-inoculated plants, respectively. Moreover, the in vitro-raised uninoculated plants also showed higher antioxidant activity

than control plants. The high concentrations of secondary metabolites (tannins, polyphenols, alkaloids, and flavonoids) may be the cause of their powerful antioxidant potential. Owing to their redox characteristics and ability to generate singlet oxygen, secondary metabolites have previously been shown to have substantial antioxidant activity and function as primary antioxidants [74,75].

4. Discussion

This study successfully established an in vitro culture of *N. jatamansi* and demonstrated that inoculation with the PGPR strains *Bacillus subtilis* and *Pseudomonas corrugata* significantly enhanced secondary metabolite accumulation and antioxidant potential in plantlets. It also promotes root development, which expands the absorptive surface area, amplifying water and mineral uptake.

Even though inoculation with *B. subtilis* and *P. corrugata* increased the antioxidant potential and phytochemical constituents, more research on the plant's essential oil is necessary to fully comprehend the role of elicitors in the synthesis of secondary metabolites. While our results confirm that *B. subtilis* and *P. corrugata* can indeed enhance phytochemical profiles and antioxidant capacity, further investigation into essential oil profiles, gene expression (transcriptomics), and metabolomic profiling would provide deeper mechanistic insights and support biotechnological applications for large-scale production. Only two strains were evaluated in this study; future research should consider a broader diversity of microbial strains, including potential synergistic consortia, to identify more effective or specialized elicitors. Furthermore, the current investigation focused on short-term in vitro responses. Long-term studies, particularly under field conditions, are essential to assess the persistence of PGPR effects on plant growth, yield, and metabolite accumulation over time.

Additionally, exploring how these biochemical PGPR traits influence key biosynthetic pathways would strengthen the understanding of plant–microbe interactions in secondary metabolism. This study may help fill the gap between the demand for and supply of raw materials and support the conservation of species in natural habitats. To further understand the production of secondary metabolites in response to PGPRs in these medicinal plants, transcriptome and metabolomics investigations should be conducted in the future.

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Abbreviations

The following abbreviations are used in this manuscript:

AAE	Ascorbic acid equivalent
AE	Atropine equivalent
BAP	6-Benzylaminopurine
CR	Critically endangered
DMRT	Duncan's multiple range test
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
IAA	Indoel-3-acetic acid
IUCN	International Union for Conservation of Nature
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
PGPR	Plant growth-promoting rhizobacteria
QE	Quercetin equivalent
TAE	Tannic acid equivalent
TDZ	Thidiazuron

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Review

Microbial Solutions in Agriculture: Enhancing Soil Health and Resilience Through Bio-Inoculants and Bioremediation

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Abstract: Soil microbes are important for maintaining agricultural ecosystems by promoting nutrient cycling, plant growth, and soil resilience. Microbial-based inoculants, such as bio-inoculants and bioremediation agents, have been identified as suitable means to promote soil health, reduce environmental deterioration, and achieve sustainable agriculture. Bio-inoculants, such as biofertilizers and biopesticides, promote nutrient availability, plant growth, and chemical input dependency reduction. Diverse microbial populations, especially plant growth-promoting bacteria (PGPB), enhance resistance by promoting a symbiotic association with plants and inducing natural resistance against insects. Bioremediation, the second significant microbial intervention, is the use of microorganisms for detoxifying and rehabilitating polluted soils. Methods effectively degrade organic pollutants, immobilize heavy metals, and mitigate the toxic effects of industrial and agricultural pollutants. Recent advances in microbial ecology and biotechnology, such as metagenomics, have transformed the knowledge of microbial soil communities, and tailor-made microbial formulations and monitoring equipment may be developed to maximize their activity. Though promising, environmental heterogeneity, scalability, and lack of field-based evidence constrain their widespread application. Multidimensional applications of microbial solutions in agroecology are explored in this review, with a focus on their potential in maintaining soil health, crop production, and environmental sustainability. It also addresses the application of bioremediation and microbial inoculants in agroecosystems and technological innovations with future research objectives. Microbial innovation to shape the soil microbiome offers a valid tool for addressing global challenges in agriculture, food security, and ecological resilience in the context of climate change.

Keywords: bio-inoculants; biofertilizers; biopesticides; climate-smart agriculture; environmental sustainability; microbial solutions; soil microbiome

1. Introduction

The process of soil development, fertility, plant growth and development, resistance to stress, nutrient cycling, and carbon sequestration are all directly or indirectly affected by the soil microbiome [1]. Nonetheless, most knowledge regarding the soil microbiome originates from research into the surface top layers, discounting the subsurface layers or subsoils. Environmental impact, new additions of organic matter, levels of nutrients, amounts of gases and moisture, and physical properties all are affected by depth in soils [2]. These soil property changes influence the soil microbial community composition and modify the functional characteristics of microorganisms [3]. We can refine the precision of terrestrial C flow models and obtain a better perception of ecosystem function by understanding the variation in microbial processes with depth in the soil [4].

In the present review, the influence of soil health on the soil microbiome is reviewed, with a specific emphasis on disparities between the very top and the very bottom subsurface horizons. An inherent quality of soil is its health, which is a collection of attributes that determine and categorize the soil [5]. It is an external quality of soil that varies with the ways individuals intend to use it, e.g., to produce recreational goods or for wildlife sustainability, agricultural yields, or watershed conservation. Future harvests will have to be doubled through sustainable methods because of rising food demands and a lack of new agricultural land development since the population of the world is expected to grow at a fast pace [6]. Another integrated strategy that can responsibly address the intrinsic and pragmatic concerns about food production is sustainable agriculture. It develops new, environmentally friendly activities by combining ecological, chemical, physical, and biological concepts [7]. Sustainability may contribute to addressing global food agricultural demands. Crop development can be supplemented by the plant rhizosphere, the very small area of soil closest to the root system, with or even without balanced or minimized pesticide inputs. Evaluation of soil health is grounded in soil quality indicators that ensure a sustainable crop production for agricultural purposes [8].

The significance of soil biota elements, including the microbial community, abundance, diversity, activity, and stability, as soil quality indicators has been demonstrated in a few studies. Besides converting nitrogen into organic and inorganic forms and affecting the plant mineral consumption, structure, and yield, the soil biota facilitates mineralization of plant residues [9]. The basic processes that confer stability and production to agroecosystems are sustained by microbial communities. Soil microbial populations like beneficial nematodes, active bacteria, and arbuscular mycorrhizal fungus (AMF) are interrelated with the crop yield, fruit quality, water storage in the soil, and nutrient cycling for differentiating between improved plant health and fertility of the soil [10]. Organic farms contain greater microbial biomass carbon in the soil compared to conventionally farmed fields, as found in a seven-year study on vegetables and field crops [11]. In mold board ploughing under organic agriculture, the mean total earthworm population was higher than reduced tillage (297 m^{-2}) [12].

Global attention should be given to enhancing or rejuvenating soil health, and the evaluation of soil health indicators will enable us to gain a deeper understanding of the ingredients that form the basis of the systems that ensure sustainable agriculture [13]. Soil microorganisms, which can modify the soil structure and nutritional content, are

adversely affected by climate change. Soil tillage, crop type, fertilizer transport, and water management are agronomic practices that alter the microclimate of agriculture and affect soil microorganisms. In agricultural environments, these practices also increase agrarian production [14]. Especially in agricultural environments, soil microorganisms play a key role in maintaining the ecological balance, promoting nutrient cycling, and supporting crop growth.

Tillage of the soil, the crop variety, transportation of fertilizer, and management of water are agronomic factors that alter agriculture's microclimate and influence agricultural soil microbes [15]. These procedures increase the agrarian yield in farm settings as well. Plant development, resistance against disease, and nutrition are promoted through bio-inoculants, which are compounds of microbes with beneficial properties increasing crop yields and soil health. They are essential to sustainable farming since they lower the use of artificial pesticides and fertilizers [16]. Legume plants and rhizobia bacteria, for example, enter symbiotic relationships that drastically lower the demand for nitrogen fertilizer by allowing nitrogen fixation. Bio-inoculants can increase the crop yield and enhance the soil structure and nutritional capacity. By enhancing the water quality and restoring the biological equilibrium, bioremediation is an environmentally sound and cost-saving approach to conventional cleanup. Microbial consortia in aquatic ecosystems break down oil spills, avoiding long-term environmental degradation and promoting marine biodiversity [17]. Bioremediation methods are increasingly being incorporated into industrial waste management programs because of their considerable reduction in pollutant levels and cleaning expenses compared to older methods [18]. Microbial solutions can revolutionize a variety of industries, spanning agricultural production, environmental cleansing, economic benefits, and sustainability over the long term, as per quantitative estimations [19]. Soil health, carbon capture, and enhanced resilience against climate change effects can be improved by farmers adopting microbially supported sustainable farming practices.

The soil microbiome refers to a sophisticated network of microbes in soils, comprising viruses, fungi, bacteria, archaea, and protozoa. Microbes are responsible for plant growth, soil fertility, nutrient cycling, and ecosystem functioning because they interact with the soil environment and among themselves [20]. Knowledge of the composition and function of the soil microbiome is vital for enhancing soil fertility, land management, and agriculture. The soil microbiome is rich in diversity, with parameters including the soil texture, water content, pH, temperature, organic matter, and vegetation influencing the soil's types of microorganisms [21]. Bacteria are the most frequent and ubiquitous microorganisms in soil, accounting for the maximum microbial biomass, with important functions like nutrient cycling, organic matter decomposition, and nitrogen fixation [22]. Fungi, archaea, and viruses also make a substantial contribution. Fungi, however, are less dominant compared to bacteria but are essential in the soil microbial community, making up most of the overall microbial biomass. Archaea play a central role in carbon and nitrogen cycling, particularly in soils with extreme characteristics such as high salinity, acidic pH values, and high temperatures. Archaea are implicated in ammonia oxidation and methanogenesis, which produce methane. Crenarchaeotes and Euryarcheotic represent some of the most prevalent phyla of archaea. Viruses are also prevalent among soil microbiomes, ranging up to 10:1 more virus particles than bacteria [23]. They can infect the bacterium and fungus, changing microbial numbers and the nutrient cycle. Protozoa and nematodes, which are two eukaryotic microbes, contribute to the soil microbiome through predation, affecting the microbial community structure and nutrient cycling.

2. Interactions Between Microorganisms and Plants in the Rhizosphere

To assist plants in receiving essential nutrients such as nitrogen, phosphate, and potassium, which are often not available due to soil limitations, rhizosphere bacteria are required [24]. Through the conversion of atmospheric nitrogen into usable ammonia, nitrogen fixation, facilitated by bacteria such as *Rhizobium* and *Frankia*, makes a nitrogen source required for growth available to the plant. Leguminous plants can gain from this process through the uptake of 50–100 kg of nitrogen per hectare per year, making the soil more fertile. The productivity of plants is enhanced when there are *Rhizobium* bacteria in the rhizosphere [25]. Phosphorus solubilization, facilitated by bacteria like *Pseudomonas* and *Bacillus*, boosts the phosphorus availability in soils where this nutrient is lacking. By producing organic acids that degrade insoluble phosphorus molecules, phosphorus-solubilizing bacteria facilitate the uptake of phosphorus by plants more efficiently. In potassium-deficient soils, in particular, rhizosphere bacteria like *Bacillus* and *Pseudomonas* facilitate the mobilization of potassium, enhancing plants' uptake of potassium.

Microorganisms present in the rhizosphere reduce disease by protecting plants from soil-borne diseases. The microbes produce antimicrobial compounds, induce systemic resistance in plants, and compete with harmful diseases for space and resources [26]. Through competition with pathogenic microorganisms for resources and space, helpful bacteria such as plant growth-promoting bacteria (PGPB) inhibit the growth and development of harmful diseases. If used on plants such as maize, wheat, and tomatoes, PGPB have the capability of reducing the incidence of diseases. PGPB also produce secondary metabolites, enzymes, and antibiotics among other antimicrobials. Fungi from the genus *Trichoderma* are capable of lowering pathogen-induced root diseases and protecting the plant against root rot diseases caused by fungi such as *Fusarium* and *Rhizoctonia* [27]. Another way in which certain rhizosphere bacteria and fungi condition the plant's immune system to respond more effectively to subsequent pathogen infections is referred to as induced systemic resistance (ISR). Quantitative studies indicate that ISR can reduce the severity of diseases such as graymold and powdery mildew in treated plants.

Through the production of vitamins, enzymes, and phytohormones, rhizosphere microorganisms, especially PGPB, play a crucial role in promoting plant growth [28]. Auxins, cytokinins, and gibberellins are some of the phytohormones that stimulate cell division, nutrient uptake, and root development. For example, indole-3-acetic acid (IAA), which is synthesized by *Azospirillum* species, increases nutrient and water uptake. In rice and maize crops, PGPB treatment can increase root biomass. Cellulases, chitinases, and phosphatases are a few enzymes that can assist in degrading organic compounds and improving nutrition availability [29]. During organic agriculture when microbial degradation makes soil nutrients accessible, it is particularly useful. Soil fertility improvement can occur with the enhancement of the decomposition of organic material by enzyme-secreting bacteria. Also, some of the rhizosphere microbes produce growth hormones and vitamins enhancing the health of plants, like folic acid and biotin, which are crucial for plant development and metabolic processes [30].

Fungi, especially arbuscular *Mycorrhizal fungi* (AMF), form symbiotic relationships with the roots of plants [31]. Some instances of the taxonomy of the AMF are *Amanita*, *Boletus*, and *Russula*, whereas *Glomus* is a well-known genus [32]. Orchid *Mycorrhizal fungi* are members of the *Rhizoctonia* genus, while ericoid *Mycorrhizal fungi* are members of the *Helotiales* genus [33]. This interaction has several outcomes. By extending fungal networks of hyphae over the soil around them, these microorganisms enable plants to take up minerals, especially phosphorus. The plant supplies the fungus with carbohydrates produced through photosynthesis and nutrient transfer by providing a greater surface

area for nutrient uptake [34]. AMF allows plants to take up micronutrients such as copper and zinc as well as phosphorus, which otherwise are hard to obtain [35]. In several crop varieties, it is estimated that AMF enhances phosphorus uptake [36]. AMF also improves water and nitrogen uptake, which makes plants drought-resistant. Studies have shown that AMF symbiotic plants are more resistant to drought, with some crops showing increases in production when under water stress. The hyphal networks formed by AMF also contribute to soil aggregation, which improves the soil structure. Plant growth is facilitated by this aggregation, which boosts air penetration and water retention. AMF-inoculated soil aggregates are believed to enhance soil porosity, which will facilitate root development and soil aeration [37].

Beneficial fungi, bacteria, and other microbes are types of microbial inoculants increasingly widely recognized as a critical component of sustainable agriculture [38]. Soil, seeds, or crops are sprayed with these beneficial microbes to enhance soil fertility, promote plant growth, prevent disease, and enhance overall crop yield. With the growing demand for environmentally friendly farming methods that reduce chemical application, microbial inoculants offer an alternative that assists farmers in soil management, increasing yields and reducing the environmental impact [39]. The effectiveness of microbial inoculants, particularly when quantitatively measured, highlights their significance as an essential part of modern farming methods.

3. Roles and Benefits of Microbial Inoculants

3.1. Enhanced Nutrient Availability

Under less-nutrient-rich soils, where plant growth is often limited by the supply of essential elements such as nitrogen and phosphorus, microbial inoculants are beneficial in enhancing the availability of nutrients. *Rhizobium* and *Azospirillum* bacteria form symbiotic relationships with legumes, fixing nitrogen from the atmosphere and converting it into ammonium, which can be utilized by plants [40]. Moreover, they reduce the dependence on synthetic nitrogen fertilizers, which reduce costs and are environmentally friendly. Microorganisms that produce organic acids, including *Pseudomonas*, *Bacillus*, and *Penicillium*, solubilize phosphorus and make it available to plants [41]. Phosphorus-solubilizing bacteria in phosphorus-deficient soils can increase phosphorus availability, which can boost the crop yield. By releasing potassium from the insoluble minerals in the soil, microbial inoculants are capable of mobilizing potassium, yet another nutrient for plants. Through research, potassium-solubilizing microbes can enhance uptake in plants such as rice and maize up to the maximum level.

3.1.1. Nitrogen Fixation (Symbiotic and Free-Living)

In symbiotic relationships with legumes and non-leguminous plants, respectively, various bacteria, primarily *Rhizobium* and *Azospirillum*, convert atmospheric nitrogen (N_2) into ammonia (NH_3) or other forms that can be readily used by plants [42]. Because nitrogen is often the most limiting factor in soils, nitrogen fixing is a critical process. In agriculture, where there is excessive consumption of artificial nitrogen fertilizers and resultant adverse effects on the environment, such as acidification of soils and greenhouse gases, this becomes very crucial. *Rhizobium* bacteria induce the formation of nodules in legume root cells like peas and soybeans [43]. The bacteria in the nodules convert nitrogen in the atmosphere to ammonium ions, which are later absorbed by plants to develop proteins, amino acids, and other essential plant constituents. *Azospirillum* attaches itself to grasses like wheat and maize, fixing nitrogen in the rhizosphere, particularly in places where nitrogen is limited. *Rhizobium* inoculation can increase nitrogen fixation the most, which increases legume

crop yields [44]. In nitrogen-deficient conditions, *Azospirillum* inoculation can increase the nitrogen uptake, boosting the plant biomass and favoring healthier development.

3.1.2. Phosphorus Solubilization

Phosphorus is often present in soils in forms that are unavailable to plants, particularly as insoluble phosphates. Microbial inoculants, particularly phosphorus-solubilizing bacteria (PSB) such as *Bacillus* and *Pseudomonas*, secrete organic acids, phosphatases, and other metabolites that hydrolyze these insoluble phosphorus compounds into plant-available forms [45]. Through the release of protons (H^+) or organic acids into the soil, these bacteria facilitate the breakdown of the bonds that phosphorus shares with other minerals, thus making it better available to the roots of plants. Phosphate ions are liberated when organic phosphorus molecules are hydrolyzed by the enzymatic synthesis of phosphatases. Under phosphorus-deficient conditions, use of phosphorus-solubilizing bacteria can increase the availability of phosphorus, and in many cases, this leads to an increase in crop yield [46]. These bacteria, through the conversion of bound phosphorus into a bioavailable form, could raise the phosphorus uptake in soils with high phosphorus.

3.1.3. Potassium Mobilization

Even though potassium is required for plant growth and health, it often occurs in soil in an insoluble state unavailable to plants. Through the formation of organic acids and other metabolites, some microbial inoculants, e.g., *Bacillus* and *Pseudomonas*, can make potassium soluble by mobilizing potassium from soil minerals and enhancing plant availability [47]. By dissolving insoluble potassium salts or potassium-bearing minerals in the soil, these microorganisms increase the amount of potassium that is available. In potassium-deficient soils, the application of microorganisms that solubilize potassium can increase absorption. Compared to non-inoculated plants, potassium solubilization can lead to the maximum increase in growth and yield in certain crops, like rice and maize [48].

Microbial inoculants not only enhance the pool of nutrients but also induce plant growth directly via the synthesis of growth-regulating substances like vitamins, enzymes, and phytohormones (auxins, cytokinins, and gibberellins).

3.2. Phytohormone Production

Auxins, cytokinins, and gibberellins are some of the plant growth hormones that are synthesized by many beneficial microorganisms, particularly PGPB, which act to directly stimulate growth in plants by influencing root initiation, cell division, and plant metabolism [49,50]. By promoting elongation and branching in roots, auxins such as indole-3-acetic acid (IAA) enhance a plant's ability to gain water and nutrients from the soil. Cytokinins induce faster plant growth by encouraging cell division and shoot development. Gibberellins facilitate stem elongation and overall plant growth by facilitating seed germination and growth. Auxin-producing PGPB inoculation has the potential to enhance the nutrient uptake to the maximum and raise the root biomass in crops such as rice, maize, and wheat [51]. Efficiency in nutrient uptake is increased by root surface area expansion, especially in conditions of stress such as drought. Auxins, which are synthesized by some bacteria like *Azospirillum*, *Bacillus*, and *Pseudomonas*, stimulate elongation and branching of roots, enhancing the uptake of water and nutrients [52]. Based on reviews, the application of these microbes can increase root biomass, enhancing plant growth and stress resistance. In addition, rhizosphere microorganisms produce gibberellins and cytokinins that stimulate cell division and shoot elongation, which further enhances plant growth.

3.3. Vitamin and Enzyme Production

Beneficial microbes also produce vitamins, including B vitamins, and enzymes that enhance plant metabolism. For instance, *Bacillus* species can synthesize folic acid and biotin, both of which are required for plant growth [53]. Soil fertility is also enhanced with enzymes such as cellulases and phosphatases, which degrade organic matter and make nutrients available for absorption into plants. Especially in cases where nutrients are scarce, the collective effect of these compounds can promote the overall health and energy of plants.

3.4. Disease Suppression and Plant Health

The management of plant diseases caused by soil-borne pathogens necessitates the application of microbial inoculants via antibiosis, systemic resistance elicitation, and competitive exclusion, they can restrain infections via competitive exclusion in crops such as tomatoes, potatoes, and wheat, meaning root rot, blight, and wilting diseases decrease [54]. This happens as beneficial fungi and bacteria block the space and nutrient occupation required by pathogens for development in the rhizosphere. In plants such as beans and cucumbers, antimicrobial production, e.g., *Trichoderma* spp., suppresses the root disease severity by inhibiting the development of soil-borne fungal infections [55]. Certain rhizobacteria, e.g., *Bacillus* and *Pseudomonas*, induce systemic resistance (ISR), which conditions plants' immune systems to resist infections. This form of resistance offers an environmentally friendly alternative to chemical pesticides and fungicides. Microbial inoculants can contribute to the suppression of soil-borne diseases through several processes. Pathogenic organisms have less chance to become established in the rhizosphere when helpful microorganisms such as *Bacillus* and *Pseudomonas* compete with pathogenic germs for resources and space [56]. Antimicrobial metabolites such as antibiotics and antifungal compounds are produced by the most beneficial bacteria, particularly *Trichoderma* species, and directly inhibit the development of pathogens. Some bacteria present in the rhizosphere induce plants to acquire systemic resistance, which conditions their immune systems to combat infection. The ability of the plant to resist many diseases is enhanced through this resistance. *Trichoderma* inoculation enhances plant health and yield by reducing the intensity of fungal infections such as root rot [57]. It has been proven that PGPB inoculants are able to reduce the incidence of damping-off diseases in vegetables such as tomatoes and cucumbers. *Bacillus* species can reduce the severity of disease, particularly in the case of bacterial and fungal diseases such as powdery mildew and fusarium wilt.

3.5. Soil Health and Structural Improvement

Through the stimulation of soil aggregation, enhancement of organic matter decomposition, and augmentation of soil diversity, microbial inoculants can enhance soil health and structure. *Mycorrhizal fungi*, for example, can enhance soil aggregation, enhancing porosity, infiltration of water, and root penetration, thereby diminishing the susceptibility to soil erosion and enhancing the water retention capacity, in turn enhancing plant growth [58]. Microbial inoculants also hasten the decomposition of organic residues, liberating nutrients that enhance soil fertility; cellulolytic bacteria can accelerate crop residue decomposition, enhancing soil health; and favorable microorganisms can enhance soil microbial diversity, facilitating a healthier and more stable soil ecosystem [59]. Complex microbial communities can adapt more readily to environmental change, resist disease outbreaks, and maintain soil fertility in the long term. This process, by facilitating the decomposition of organic matter, enhancing soil aggregation, and enhancing soil aeration and water-holding capacity, means microbial inoculants have the potential to enhance the structure and health of the

soil. Soil aggregation extracellular polysaccharides from *Mycorrhizal fungi* and certain bacteria, including *Bacillus*, act to bind soil particles and form stable aggregates [60]. This reduces soil erosion, enhances water infiltration, and increases soil strength. Organic matter decomposition crop residues and other organic materials are decomposed into simple chemicals by microbes such as cellulolytic bacteria, which supply nutrients to the soil. More tolerant ecosystems, equipped to withstand environmental stress factors such as drought or disease, can be facilitated through enhanced microbial diversity in the soil, which is achieved by incorporating beneficial microbes [61]. Under arid conditions, *Mycorrhizal fungi* may enhance soil aggregation, enhancing root growth and water infiltration. Cellulolytic bacterial inoculation would enhance organic matter decomposition, enhancing soil fertility and speeding up nutrient cycling.

3.6. Stress Tolerance Enhancement

Microbial inoculants help plants to counteract a variety of environmental stresses, including salt, drought, and extreme temperatures. Many PGPB produce compounds such as osmo protectants, which help in the regulation of osmotic stress in plants or trigger the defense mechanisms of a plant itself [62]. Microbes produce osmo protectants, including proline and trehalose, which protect plant cells from dehydration during periods of excessive salt or drought. Microbial interactions yield heat shock proteins (HSPs) to enable plants to cope with temperature fluctuations and excessive heat stress. PGPB inoculation can enhance drought tolerance among crops, enabling them to survive prolonged water scarcity conditions [63]. Microbial inoculants have been shown to enhance the yield and water-use efficiency in salt-stressed crops through the mitigation of salinity effects on growth.

4. Types of Microbial Inoculants

Different types of microbial inoculants can be distinguished based on their composition and intended application. The following are the main categories of microbial inoculants (Table 1; Supplementary Table S1).

Table 1. Microbial inoculants' impact on agriculture, including their effects on nutrient availability, plant growth, disease suppression, and soil health.

Microbial Inoculum	Effect	Details of Effects	References
Rhizobium Nitrogen-Fixing Bacteria	Nitrogen fixation in legumes	Increase nitrogen fixation and crop yields	[64]
Azospirillum Nitrogen-Fixing Bacteria	Nitrogen fixation and plant growth	Increase nitrogen uptake and enhance root biomass	[65]
Pseudomonas (PGPB)	Phosphorus solubilization and plant growth	Increase phosphorus availability and yield	[66]
Bacillus (PGPB)	Phytohormone production and growth promotion	Increase root biomass and improve nutrient uptake	[67]
Trichoderma Fungal Inoculant	Disease suppression and growth promotion	Reduce root rot and other diseases by enhancing growth in stress conditions	[68]
Mycorrhizal fungi (AMF)	Phosphorus uptake and soil structure	Increase phosphorus uptake and improve soil aggregation	[69]
Bacillus (PGPB)	Disease suppression via ISR	Reduce disease severity and enhance resistance to fungal and bacterial diseases	[70]
Cellulolytic Bacteria	Organic matter decomposition	Accelerate decomposition and improve nutrient cycling and soil fertility	[71]
Potassium-Solubilizing Microbes	Potassium mobilization	Increase potassium uptake in potassium-deficient soils	[72]
Cellulolytic Bacteria	Organic matter decomposition	Accelerate decomposition and improve nutrient cycling and soil fertility	[71]
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5. Microbial Bioremediation: Restoring Soil Health

5.1. Degradation of Organic Pollutants

A very common bioremediation process is the degradation of organic pollutants, such as hydrocarbons, insecticides, and solvents. Enzymes in microorganisms such as bacteria, fungi, and actinomycetes can break down large organic molecules into simpler, less toxic forms. This can occur either aerobically (with oxygen) or anaerobically (without oxygen) depending on the contaminant and microbial species [73]. When organic pollutants such as petroleum hydrocarbons are aerobically degraded, oxygen is typically employed to oxidize the pollutants and convert them into water and carbon dioxide (CO_2). Reductive dichlorination is employed by specialized bacteria to degrade organic pollutants such as chlorinated solvents in anaerobic conditions, often producing non-toxic end products such as ethene. Quantitative data confirm the efficacy of bioremediation in the degradation of organic pollutants. For example, it has been reported that fungi such as *Phanerochaete chrysosporium* can degrade man-made colors and pesticides in contaminated soils [74], and that bacteria such as *Pseudomonas putida* and *Mycobacterium* species can degrade petroleum hydrocarbons within weeks [75]. By stimulating natural microbial activities to eliminate toxins from the environment, bioremediation not only reduces the toxicity of pollutants but can also potentially heal ecosystems [76].

5.2. Detoxification of Heavy Metals Through Biosorption and Bioaccumulation

Since heavy metals are environmentally persistent and toxic to living organisms, there is increasing concern about heavy metal contamination. The two major operations carried out under heavy metal bioremediation are biosorption and bioaccumulation. However, many other mechanisms of detoxification are possible in a microbial cell (Figure 1; Supplementary Table S2). The procedure by which bacteria and fungi adsorb metal ions on their cell wall or extracellular material is referred to as biosorption [77]. This process is often a passive accumulation process and does not require the bacteria to metabolize the metals. Bioaccumulation, in contrast, is where the heavy metals are actively taken in and concentrated inside microbial cells. Some species of *Bacillus* and *Pseudomonas* can bioaccumulate lead (Pb), cadmium (Cd), and mercury (Hg) from contaminated environments, storing them in non-toxic forms [78]. These transformations play a crucial role in microbial detoxification and bioremediation strategies. For example, mercury can be enzymatically reduced to elemental mercury, which is volatile and less reactive, or it can be precipitated as mercuric sulfide, an insoluble and stable compound. Cadmium may be sequestered intracellularly through binding with metallothionein, like proteins, or precipitated as cadmium phosphate, reducing its mobility and toxicity [78]. Similarly, lead can be immobilized through the formation of insoluble compounds, such as lead phosphate or lead sulfide. These biochemical conversions as highlighted in the review by Alotaibi et al., 2021, along with the functional versatility of these microbial genera in mitigating heavy metal contamination through bioinformation and bioaccumulation mechanisms [78]. Heavy metal amounts in polluted zones may be significantly reduced thanks to the ability of microorganisms to accumulate and detoxify them. For instance, it has been shown that *Sphingomonas* species can bioaccumulate as much as or at higher levels than chromium in industrial effluent, while *Pseudomonas putida* could accumulate as much dry weight as copper ions in contaminated water [79]. The concentration of heavy metals in contaminated areas is, at times, brought down by maximally using such microbes in the process of bioremediation, making the environment healthier for both flora and fauna.

5.3. Transformation of Persistent Organic Pollutants (POPs)

Toxic chemicals referred to as persistent organic pollutants (POPs) tend to accumulate in living organisms, are resistant to breakdown in the environment, and cause severe health risks [80]. Polychlorinated biphenyls (PCBs), dioxins, and pesticides like DDT are some examples of POPs. Microorganisms with the ability to change the chemical structure of POPs can modify them via bioremediation, reducing their toxicity and enhancing their biodegradability [80]. The stable, complex structures of POPs can be degraded into less toxic intermediates by enzymatic reactions called biotransformation, which is a function of certain bacteria and fungi. Reductive dichlorination, which converts chlorinated compounds such as PCBs and DDT into less chlorinated or non-chlorinated products, is another reaction that bacteria can sometimes carry out [81]. *Bacillus* and *Mycobacterium* species have been used to break down organochlorine pesticides, including DDT, in contaminated soils, whereas *Dehalococcoides* species have been shown to dechlorinate as much as PCB congeners in contaminated sediments [82]. Such types of bioremediation methods help reduce the long-term impacts of POPs on ecosystems and human health as well as reduce their persistence in the environment.

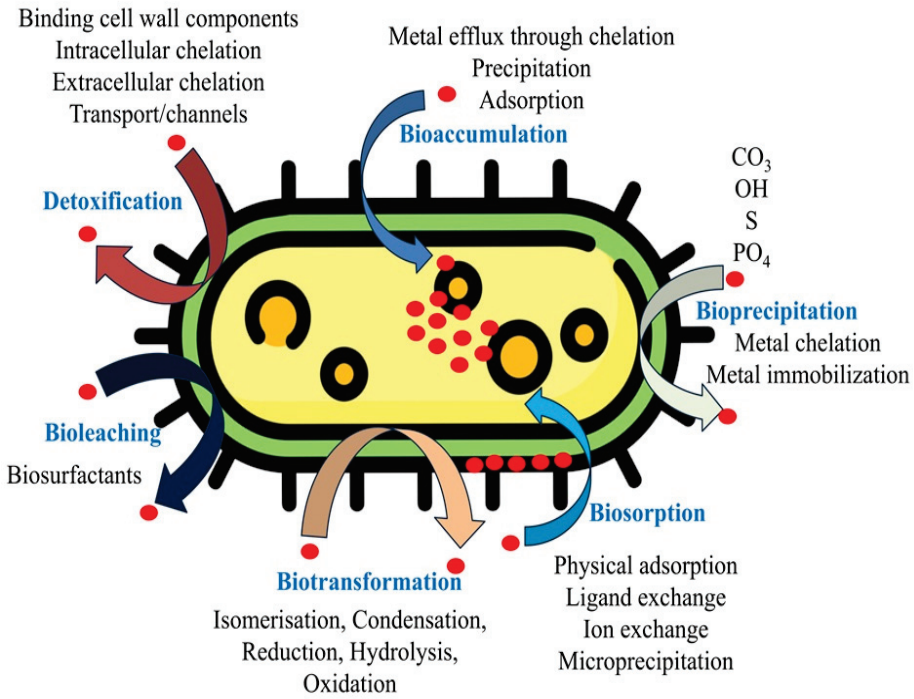

Figure 1. Heavy metal detoxification mechanisms by a bacterial cell (Supplementary Table S2).

6. Applications for Bioremediation in Agricultural Soils

6.1. Cleaning up Pesticide-Contaminated Soils

Though essential for crop pest management, the application of pesticides in agriculture often leads to the contamination of water and soil resources. Pesticides such as organochlorines (e.g., DDT), organophosphates, and carbamates are examples that can persist for many years in the soil, causing damage to soil microorganisms, reducing biodiversity, and affecting the crop yield [83]. Through the action of microorganisms that can degrade or transform pesticides into harmless byproducts, bioremediation offers a sustainable method for remediating pesticide-polluted soils. Microbial species such as *Pseudomonas*, *Bacillus*, and *Rhodococcus* are known to metabolize a wide range of pesticides by dissolving their complex chemical structures [84]. The microorganisms dechlorinate

or hydrolyze pesticides, reducing their toxicity, through the action of enzymes such as esterases and dehalogenases. For instance, *Pseudomonas putida* can significantly reduce the concentration of organophosphate pesticides in the soil by degrading them within a few weeks [85]. Apart from detoxifying the toxins, bioremediation restores the microbiome of the soil, providing an environment that is more conducive to microbial diversity and plant growth (Table 2).

Table 2. Microbiomes and their functions and details.

Microbiomes	Functions/Details	References
Microbial Biomass	Soil contains between 10^7 and 10^9 microbial cells per gram of soil.	[86]
Bacterial Biomass	Bacteria make up most of the total microbial biomass in the soil.	[87]
Fungal Biomass	Microbial biomass.	[88]
Bacterial Phyla	Proteobacteria: Dominant in nutrient cycling and organic matter degradation. Firmicutes: Important for breaking down complex organic molecules. Actinobacteria: Significant in organic matter decomposition and antibiotic production. Acidobacteria: Key players in acidic soil environment and nutrient cycling. Bacteroidetes: Degrade complex organic compounds.	[89]
Fungal Phyla	Ascomycota: Important in decomposing plant material. Basidiomycota: Key in forming mycorrhizal associations and decaying complex materials. Zygomycota: Important for nutrient cycling and plant symbiosis.	[90]
Archaea	Archaea represent a smaller portion but are critical in methanogenesis and ammonia oxidation.	[91]
Viruses in Soil	Viruses can outnumber bacteria by a ratio of 10:1 and regulate microbial diversity.	[92]
Protozoa and Nematodes	Protozoa and nematodes are important predators of soil bacteria and fungi.	[93]
Microbial Contributions to Soil Health and Nutrient Cycling		
Microbial Role in Nitrogen Fixation	Nitrogen fixation bacteria, e.g., *Rhizobium*, *Frankia*, contribute most of total nitrogen input.	[94]
Organic Matter Decomposition Rate	Microbial decomposition rates in temperate soils.	[95]
Soil Carbon Storage	Total soil organic carbon.	[96]
Phosphorus Cycling	Phosphorus-solubilizing bacteria, e.g., *Pseudomonas*, help convert insoluble phosphorus into bioavailable forms.	[97]
Soil Aggregate Formation	Fungi produce extracellular polysaccharides to bind soil particles, improving soil aggregation.	[98]
Soil Disease Suppression	Beneficial microbes can produce antibiotics or enzymes that suppress soil-borne pathogens.	[99]
Plant Growth Promotion	Plant growth-promoting bacteria (PGPB) produce phytochromes, e.g., auxins, cytokinins, that enhance root growth.	[100]

6.2. Managing Industrial Effluents and Wastewater in Agroecosystems

Wastewater and toxic effluents are often discharged into the environment due to industrial activities, destroying nearby agricultural lands [101,102]. Heavy metals, oils, organic solvents, and other toxic substances that could harm plants and lower the soil quality could be contained in these effluents [103]. By utilizing microorganisms that could either degrade or immobilize the pollutants, bioremediation provides an effective means of managing such pollutants, minimizing their toxicity. Petroleum hydrocarbons, for example, can be degraded into simpler non-toxic compounds by hydrocarbon-degrading bacteria and fungi that act on hydrocarbon components of industrial waste [104,105]. Additionally, through a process such as biosorption and bioaccumulation, bacteria such as *Bacillus* and *Pseudomonas* efficiently remove heavy metals such as lead, mercury, and cadmium from polluted water and soil. Aside from sanitizing the effluents, microorganisms decrease the bioavailability of toxic metals so that plants and animals cannot take them up. Biological ecosystems in agriculture can benefit from reduced levels of chemical pollution by using bioremediation methods, which will lead to better soil and increased crop production [106].

6.3. Role in Reducing Greenhouse Gas Emissions

Methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2) are the primary greenhouse gases (GHGs) emitted by the agricultural sector [107]. Rice paddies emit methane, but waste management and commercial fertilizers emit nitrous oxide. By increasing microbial processes that either oxidize or reduce GHGs, bioremediation can be instrumental in reducing these emissions. Denitrifying soil nitrates to nitrogen gas (N_2) in anaerobic conditions, for instance, certain soil microbes, including denitrifying bacteria, can effectively limit the release of this potent greenhouse gas [108]. Methane release in rice paddies may also be limited by using bioremediation methods involving methanotrophic bacteria, which utilize methane as a source of energy. Second, enhancing soil organic matter by adding organic amendments (e.g., compost) will stimulate carbon-sequestering microbial communities, decreasing atmospheric CO_2 levels [109]. As well as reducing GHG emissions, these microbial applications increase crop yields, fertility, and soil health.

6.4. Integration of Microbial Solutions into Agroecosystems

Agroecosystem incorporation of microbial solutions presents an integrated strategy to achieve profitable and sustainable agricultural practices [110]. Farmers may enhance crop growth, reduce environmental contamination, and enhance soil integrity by integrating bio-inoculants and bioremediation strategies. Apart from mitigating prevailing problems such as insect infestations, soil contamination, and nutrient shortages, this integrated strategy towards soil management enhances ecological sustainability in the long term [111].

6.5. Combining Bio-Inoculants and Bioremediation for Holistic Soil Management

There is substantial potential for enhancing soil fertility and productivity by using the dual approach of bio-inoculants and bioremediation. *Mycorrhizal fungi*, nitrogen-fixing bacteria, and plant-growth-promoting rhizobacteria are some examples of bio-inoculants that could promote the growth of plants, enhance nutrient uptake, and reduce the requirement for synthetic fertilizers [112]. But bioremediation techniques can eliminate toxins from the soil and re-establish its microbial equilibrium through the application of microorganisms to detoxify heavy metals and degrade organic pollutants. Practically, this could be achieved through the application of fungi such as Trichoderma, which degrades pesticide residues in polluted soils, in combination with *Rhizobium* or *Azospirillum* for nitrogen fixation, which can enhance soil fertility [113], promote healthier plant growth, and minimize the environmental impact of agricultural practices. When these two approaches are combined, they create a synergistic effect that not only stimulates plant growth but also purifies the soil of toxic chemicals, minimizes nutrient leaching, and revives the microbial ecosystem.

6.6. Strategies for Incorporating Microbial Products into Integrated Pest and Nutrient Management Systems

Through the successful integration of microbial solutions into nutrient management and integrated pest management (IPM) systems, agroecosystem issues can be handled in a sustainable way. Microbial products such as biopesticides *Bacillus thuringiensis*, *Beauveria bassiana*, and *Trichoderma* spp. represent a natural alternative to traditional pesticides in integrated pest control [114,115]. Without posing a threat to the ecosystem or beneficial organisms in the soil, these microbial bioagents eradicate or inhibit pest and disease development. For example, *Trichoderma* species are very effective in controlling fungal diseases in the soil, while *Bacillus thuringiensis* is widely used to control insect pests like caterpillars. These microbial biopesticides reduce the use of traditional pesticides, which can adversely affect biodiversity, soil quality, and human health [116]. Microbial inoculants play a signifi-

cant role in enhancing the availability of essential nutrients such as potassium, phosphorus, and nitrogen in nutrient supply systems. Fertilization programs may utilize microorganisms that fix nitrogen such as *Rhizobium* and *Azotobacter* or phosphorus solubilizers such as *Pseudomonas* and *Bacillus* species to reduce nutrient runoff into adjacent ecosystems and reduce the need for chemical fertilizers [117–119]. These microbial metabolites help promote the sustainability of agroecosystems through enhanced soil aggregation, decomposition of organic matter, and water absorption as well as providing nutrition to plants.

6.7. Synergistic Effects of Diverse Microbial Communities over Single Strains

The synergistic effect that multiple strains can exert on the use of a single microbial strain is one of the primary benefits of adding various microbial communities to agroecosystems. While individual microbial species may offer specific benefits, e.g., disease control or nitrogen fixation, a diverse community of microorganisms can serve the environment more generally and strongly [120]. Collectively, these communities perform numerous functions that enhance the balance and functionality of the agroecosystem, including nutrient cycling, organic matter decomposition, disease suppression, and soil structural development. Applying a mixed inoculant that harbors both *Mycorrhizal fungi* and nitrogen-fixing bacteria might be more effective than the application of each organism separately [121]. The *Mycorrhizal fungi* enhance the water uptake and nutrient acquisition, especially phosphorus, and the nitrogen-fixing bacteria enhance the pool of available nitrogen. Bacteria also aid in maintaining ecosystem stability by protecting each other from abiotic stresses such as drought or temperature fluctuations. By stimulating competition among beneficial microorganisms and harmful pathogens, the combination of diverse microbial species further enhances disease suppression [122]. Soil diversity, plant resistance against pests and diseases, and total agricultural production may all be enhanced by richly diverse microbial populations.

7. Advances in Soil Microbiome Research and Technology

7.1. Metagenomics and Microbial Ecology

Metagenomics is transforming our comprehension of microbial diversity and the community composition, especially for soil communities. Metagenomics examines genetic material directly from environmental samples, which enables scientists to obtain a broad picture of microbial communities without the necessity of cultivating microorganisms [123]. Metagenomics enables the development of novel microbial inoculants by providing comprehensive insights into the diversity, structure, and functional potential of soil and rhizosphere microbiomes without the need for cultivation. Through high-throughput sequencing, it identifies genes responsible for beneficial plant traits such as nitrogen fixation, phosphate solubilization, hormone production, and stress tolerance. This allows researchers to discover unculturable or previously unknown microbial taxa and assess their functional roles, making it possible to select, design, or engineer microbial consortia with synergistic capabilities tailored to specific crops or environments. Consequentially, metagenomics facilities the formulation of targeted efficient and sustainable biofertilizers and soil inoculants that enhance the plant growth nutrient uptake and resilience to stress. Metagenomic analysis detects intricate interactions that form microbial communities, including bacteria, viruses, fungus, archaea, and protists. It also reveals the functional potential of such communities by discovering genes that are responsible for vital processes such as nutrient cycling, disease suppression, and plant growth promotion. The most fascinating use of metagenomics in microbial ecology is the identification of functional genes with possible agricultural uses [124]. Such genes, involved in nitrogen fixation, phosphate solubilization, and hormone production, can be utilized to enhance sustainable

agriculture (Table 3). Metagenomics also holds the potential to create bio-based fertilizers and soil inoculants to allow microorganisms to exert beneficial processes. Discovery of stress tolerance or disease resistance genes in plants would result in more resilient crops, which would mitigate the environmental effects of traditional agriculture [125].

Table 3. Genes involved in nitrogen fixation, phosphate solubilization, and plant hormone production.

Gene	Full Name	Function	Notes	References
Nitrogen fixation genes				
nifH	Nitrogenase iron protein gene	Encodes dinitrogenase reductase, essential for nitrogen fixation	Common marker gene for diazotrophs (N_2-fixing bacteria); widely used in metagenomic studies	[126–128]
nifD	Nitrogenase alpha subunit gene	Encodes one of the catalytic components of nitrogenase	Works with *nifK* to form dinitrogenase	[129–131]
nifK	Nitrogenase beta subunit gene	Encodes another catalytic subunit of nitrogenase	Required for the full nitrogenase complex function	[130,132,133]
nifA	Nitrogen fixation regulatory protein A	Activates transcription of nitrogen fixation genes	Regulates *nif* gene cluster expression under low nitrogen	[134–136]
nifL	Nitrogen fixation regulatory protein L	Acts as a negative regulator of *nif* genes in the presence of oxygen or ammonia	Balances nitrogen fixation activity with environmental conditions	[134,137]
Phosphate solubilization genes				
gcd	Glucose dehydrogenase gene	Oxidizes glucose to gluconic acid	Gluconic acid solubilizes insoluble phosphates in the soil	[138–140]
phyA*/*appA	Phytase genes	Hydrolyze phytic acid to release inorganic phosphate	Important in mobilizing organic phosphorus	[141–143]
phoA	Alkaline phosphatase gene	Hydrolyzes organic phosphates under alkaline conditions	Enhances phosphate availability	[144–146]
phoD	Alkaline phosphatase D gene	Another alkaline phosphatase with high activity in soils	Frequently found in plant rhizospheres; good marker for phosphorus cycling potential	[147–149]
Hormone production genes				
ipdC	Indole-3-pyruvate decarboxylase gene	Catalyzes conversion of indole-3-pyruvate to indole-3-acetaldehyde in IAA biosynthesis	Key gene in the tryptophan-dependent IAA pathway in PGPR	[150–152]
iaaM	Tryptophan monooxygenase gene	Converts tryptophan to indole-3-acetamide	Part of the IAM (indole-3-acetamide) pathway in IAA synthesis	[153–155]
iaaH	Indole-3-acetamide hydrolase gene	Converts indole-3-acetamide to IAA	Works in tandem with *iaaM*	[153,156,157]
amiE	Amidase gene	Involved in auxin (IAA) biosynthesis	Associated with tryptophan metabolism	[158–161]
Ipt	Isopentenyl transferase gene	Involved in cytokinin biosynthesis	Catalyzes the first step in cytokinin formation; often found in endophytes	[162–164]
cps	Copalyl diphosphate synthase gene	Involved in gibberellin biosynthesis	Key enzyme in diterpenoid hormone production like GA	[165–167]
ks	ent-Kaurene synthase gene	Converts CPP to ent-kaurene, a GA precursor	Works with cps in GA biosynthetic pathway	[165,168]
ga20ox	Gibberellin 20-oxidase gene	Catalyzes formation of bioactive gibberellins	Important for growth regulation	[169–171]
acdS	1-Aminocyclopropane-1-carboxylate deaminase gene	Breaks down ACC, a precursor to ethylene	Reduces stress ethylene in plants, promoting root elongation and stress tolerance	[172–174]

7.2. Biotechnology in Microbial Product Development

Biotechnology has played a major role in the production of microbial products, most importantly stress-resistant strains and efficient delivery systems for microbial inoculants [175]. Genetic engineering techniques can increase microbial strains' tolerance to environmental stresses such as drought, salt, high temperatures, and changes in soil pH. Modified microorganisms can improve sustainable agricultural practices by reducing the negative effects on plant growth. Stress-tolerant strains of *Rhizobium* or *Azotobacter* have been bred to enhance nitrogen fixation in nutrient-poor soils [176]. To ensure the efficacy of these biotechnologically modified microorganisms, innovative formulation and delivery systems for microbial inoculants must be formulated in parallel. Traditional microbial inoculants tend to lack viability, shelf life, and soil environment establishment. Biotechnological advancements are directed towards encapsulating microbial strains within protective vehicles such as hydrogels, nanoparticles, or bio-based matrices [177]. Controlled-release methods are also in progress to facilitate effective delivery to the root zone. Such biotechnological advances are developing more robust and potent microbial products that are poised to substantially influence sustainable agriculture.

7.3. Monitoring and Assessment Tools

Monitoring and evaluation tools are essential for establishing soil health and microbial activity. Biomarkers and molecular tools, including qPCR, metagenomics, and DNA/RNA sequencing, are the leaders in this area [178]. These methods precisely detect and measure microbial abundance and functional genes and yield excellent information on microbial diversity, community composition, and potential functions. Biomarkers are molecules or genes that indicate microbial activity, health, and ecosystem functioning [179]. For example, particular functional genes such as nitrogenase or phosphatase genes are suitable as microbial activity biomarkers of essential processes in soil fertility and plant growth. The technology further enables microbial activity monitoring using gene expression assessment, meta transcriptomics, and enzymatic activity meta proteomics [180]. Molecular biosensors and probes are being created to detect microbial activities or species linked to the health of soil so that farmers and soil scientists can quickly diagnose. These new monitoring techniques can inform the creation of targeted plans for enhancing soil health and sustainability.

8. Challenges and Future Perspectives

Soil–microbe–plant interaction agriculture is confronted with various challenges because of an unawareness of the complex interactions between soil, microbes, plants, and the environment [181]. The specific mechanisms of these processes are under study, and the success of microbial treatments is soil- and crop-dependent locally. Standard protocols for applying and validating microbial treatments are another serious challenge. The scalability of microbial solutions to large-scale agricultural systems is still challenging owing to aspects like microbial viability upon storage, inconsistent performance between different environments, and variability in microbial product compositions [182]. Lack of proper regulations and lengthy approval procedures might discourage farmers from embracing microbial solutions. There is a need for interdisciplinary research to create more resilient, scalable, and locally applicable microbial products. Socioeconomic determinants must be taken into consideration, as knowledge of farmer conduct, economic viability, and effects of microbial solutions on yields and profitability is key to their uptake at the large scale [183]. Policy support for regulation and marketing of microbial products is required, with governments and intergovernmental organizations developing policies in

favor of research and development on microbial biotechnology, offering fiscal incentives for eco-friendly agriculture operations, and putting regulations in place for the proper use of microbial inoculants in the field [184]. Microbial solutions have strong potential in climate-smart agriculture because they could counteract climate change-induced increases in droughts, floods, and temperature extremes. Nitrogen-fixing or phosphorus-solubilizing soil bacteria could minimize the use of chemical fertilizers, and drought-tolerant microbial isolates could enhance water-use efficiency in arid areas [185]. Soil microbes have the potential to mitigate climate change through carbon sequestration mechanisms. Regulatory frameworks and policy support are essential to streamline approval processes for microbial solutions, ensuring their safe and effective implementation in large-scale agriculture and production. Additionally, addressing stakeholder concerns, including farmer adoption and economic viability, will be key to accelerating the use of microbial innovations in sustainable farming.

9. Conclusions

Microbial technologies can enhance soil health and decrease the dependency on chemical fertilizers. Soil fertility can be increased by adding beneficial microbes such as *Mycorrhizal fungi*, phosphorus-solubilizing bacteria, and nitrogen-fixing bacteria, raising yields. Bio-inoculants can also elevate stress tolerance towards salinity and drought, while drought-resistant varieties can improve the efficiency of water use. Bacteria in soil have an important role to play in bioremediation, decontaminating toxins such as pesticides, heavy metals, and petroleum hydrocarbons, enriching the quality of soil, and lessening pollution. Certain bacteria have the capability of producing the maximum breakdown of residues from pesticides in polluted soils in weeks. Microbial solutions assist in the establishment of stable organic carbon in soils, which enriches the soil structure and water storage capacity. Yet, there are obstacles like standard application routes and a microbial efficiency difference that must be overcome prior to extensive application. Microbial solutions should become a central pillar of sustainable agricultural practices, as agriculture shifts towards more climate-responsible strategies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/bacteria4030028/s1, Table S1: Comprehensive classification of microbial inoculants based on composition, species diversity, and functional purpose; Table S2: Quantitative Comparison Between Bioprecipitation and Biosorption in Heavy Metal Removal.

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Abbreviations

PGPB	Plant growth-promoting bacteria
C	Carbon
N	Nitrogen
P	Phosphorus
AMF	Arbuscular mycorrhizal fungus
ISR	Induced systemic resistance
IAA	Indole-3-acetic acid
NH_3	Ammonia
N_2	Nitrogen
PSB	Phosphorus-solubilizing bacteria
H^+	Protons
HSPs	Heat shock proteins
CO_2	Carbon dioxide
Pb	Lead
Cd	Cadmium
Hg	Mercury
POPs	Persistent organic pollutants
DDT	Dichloro-diphenyl-trichloroethane
CH_4	Methane
N_2O	Nitrous oxide
GHGs	Greenhouse gases
IPM	Integrated pest management

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Review

Microbial Antagonists for the Control of Plant Diseases in *Solanaceae* Crops: Current Status, Challenges, and Global Perspectives

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Abstract: Postharvest losses of *Solanaceae* crops, which include potatoes (*Solanum tuberosum*), tomatoes (*Solanum lycopersicum*), bell peppers (*Capsicum annuum*), and others, are one of the major challenges in agriculture throughout the world, impacting food security and economic viability. Agrochemicals have been successfully employed to prevent postharvest losses in agriculture. However, the excessive use of agrochemicals may cause detrimental effects on consumer health, the emergence of pesticide-resistant pathogens, increased restrictions on existing pesticides, environmental harm, and the decline of beneficial microorganisms, such as natural antagonists to pests and pathogens. Hence, there is a need to search for a safer and more environmentally friendly alternative. Microbial antagonists have gained more attention in recent years as substitutes for the management of pests and pathogens because they minimize the excessive applications of toxic substances while providing a sustainable approach to plant health management. However, more research is required to make microbial agents more stable and effective and less toxic before they can be used in commercial settings. Therefore, research is being conducted to develop new biological control agents and obtain knowledge of the mechanisms of action that underlie biological disease control. To accomplish this objective, the review aims to investigate microbial antagonists' modes of action, potential future applications for biological control agents, and difficulties encountered during the commercialization process. We also highlight earlier publications on the function of microbial biological control agents against postharvest crop diseases. Therefore, we can emphasize that the prospects for biological control are promising and that the use of biological control agents to control crop diseases can benefit the environment.

Keywords: postharvest diseases; microbial antagonist; sustainable management; *Solanaceae* crops

1. Introduction

Agriculture is the backbone of the economy in Africa, contributing significantly to the gross domestic product (GDP) across the continent [1]. Potatoes from the family *Solanaceae*, are the fourth largest food crop [2]; in terms of food consumption [3,4], they are a crop of global importance for food [5] and are consumed by more than one billion people [6]. Nutritionally and financially, the cultivation of potatoes is worthy of attention from the world because they contain vitamins and minerals and are consumed daily [7]. Although potatoes can be stored for a long time compared to other crops, potato tubers can be

constrained by preharvest factors and postharvest losses due to postharvest diseases [8]. Preharvest factors include any conditions or activities that affect crops before harvesting [9]. These factors include inappropriate cultural practices, nutritional imbalances, genetic composition, harvesting methods, quality of water for irrigation, microbial invasion, insect pest inoculum remnants, and more [10,11].

Postharvest diseases are infections that occur in fruits, vegetables, and seeds after they have been harvested [12] and are mainly caused by phytopathogens [13]. These organisms include oomycetes, fungi, bacteria, nematodes, and viruses that cause diseases like warts, powdery scabs, and late and early blight. These organisms pose a serious threat to crop productivity worldwide [14]. Phytopathogens from *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp., *Penicillium* sp., and *Pseudomonas* sp. produce toxigenic metabolites (citrinin, alternariol, toxoflavin, fumonisin, and coronatine) [15], which compromise the quality and usability of harvested crops, contributing to the consumer's final decision to eat or reject the food [16]. This is why effective protection against pathogens is so important.

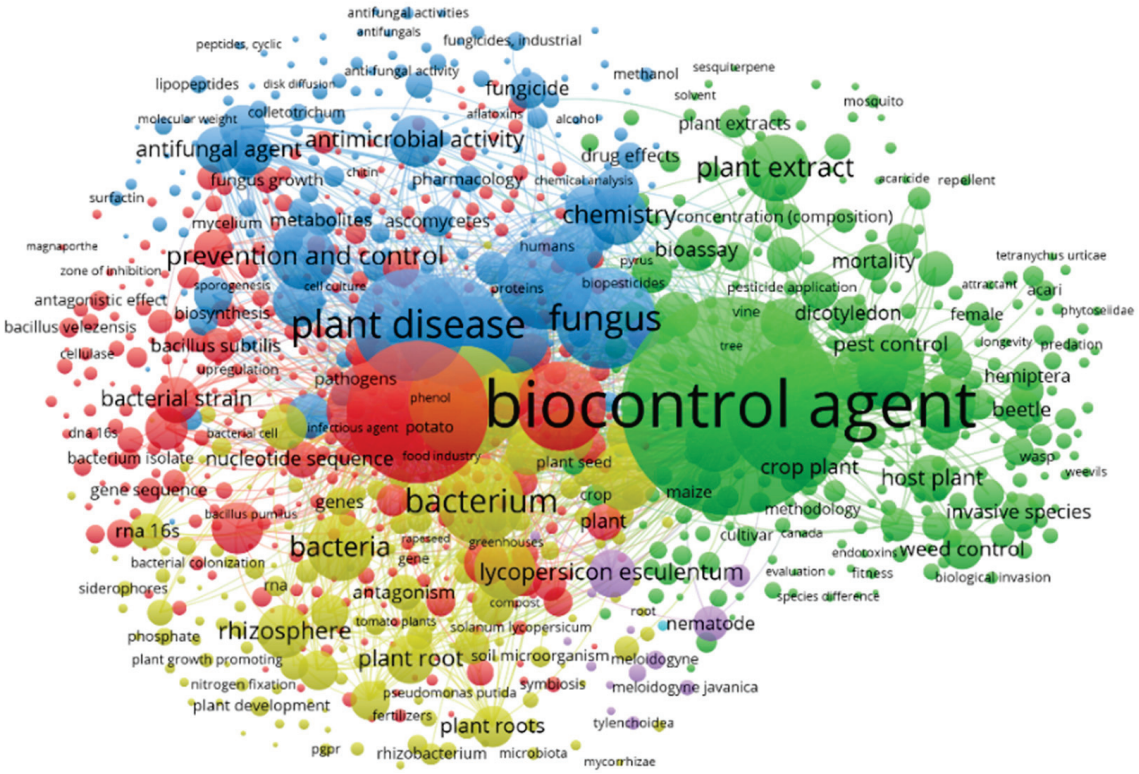
Agrochemicals have been successfully employed to reduce postharvest pathogens [17]. However, reducing agricultural production inputs like agrochemicals is of great importance, while maintaining a lucrative yield of high-quality goods is becoming more and more necessary because of the global sustainability agenda [18].

Due to growing concerns about the impact of agrochemicals on the environment, crop quality, nutrient constituents, the development of pesticide resistance in pests, and consumer health, farmers are eager to seek solutions for management practices other than agrochemicals. South Africa, as the largest agrochemical user in sub-Saharan Africa, with over 3000 registered pesticide products [19], has started to ban some of the registered products like glyphosate-based herbicides, triazine herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D), and chlorpyrifos, among others [20]. The recent trend is now shifting toward safer and more eco-friendly alternatives, such as the use of biological control microorganisms, natural compounds (essential oils and phytohormones), and physical treatments (advanced oxidation process) [21], to control postharvest diseases and decay without contaminating the environment [22]. Biological control agents (BCAs) have been regarded as a promising alternative to conventional pesticides [23], with a wide array of microbial antagonists being developed in the past few decades for the management of fungal and bacterial diseases [24,25]. Biological control agents, also known as antagonists, refer to the use of microorganisms to control insects, weeds, and pathogens that affect plants [26,27].

Evidence has shown a significant increase in the number of studies conducted between 2005 and 2025 regarding bacterial and fungal BCAs [14,28,29]. When the term "Biological control agents" was added to a Google Scholar search with the keyword "Plants", the number of records retrieved was roughly 6218 (2015–2025). The bibliometric data were extracted from the SCOPUS database (https://www.scopus.com/ (accessed on 10 January 2025)) and constructed using the VOS viewer processing software (v1.6.9., Leiden University, Leiden, The Netherlands) (Figure 1a). The network analysis showed the distribution of articles related to BCAs in plants. The network analysis was reduced to BCA use in *Solanaceae* species, which showed 132 articles with the following keywords: *Solanales*, *Solanaceae*, invasive species, Anthonomus, biological control, *Solanum maritium*, *Solanum melongena*, *Lycopersicon esculentum*, *Solanum varium*, *Capsicum annuum*, *Solanum tuberosum*, and South Africa (Figure 1b). While South Africa focuses on the development and use of BCAs as natural enemies to invasive weeds [29,30], the Scopus database showed that most published manuscripts on BCAs are from China [31], with 1247 (Figure 1c). The United States of America (USA), India, Italy, Brazil, and Spain were also ranked as the top leading countries after China in postharvest research involving BCAs based on the number

of research papers published and uploaded to Scopus since 2015–2025, as indicated by the extracted and screened document in Figure 1c.

(**a**)



(**b**)

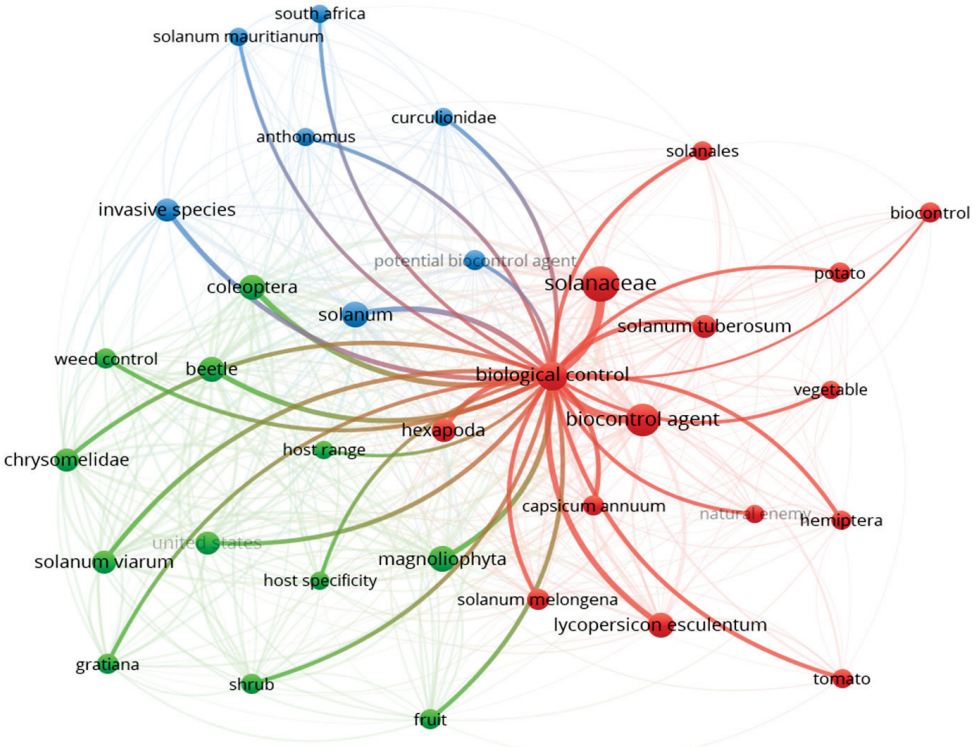


Figure 1. *Cont.*

(**c**)

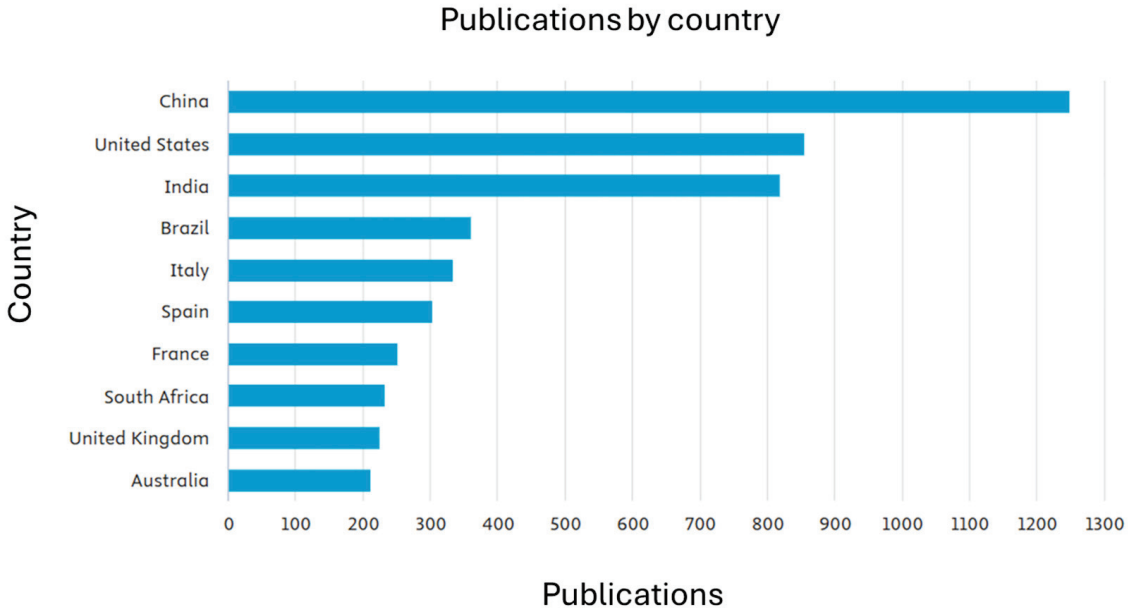


Figure 1. Bibliometric analysis of 6218 articles published on biological control according to the Scopus database using specific keywords, such as "Biological control agents" AND "Plants" (**a**), which revealed the network relationship between keywords found on *Solanaceae* (**b**) and showed a comprehensive perspective of the current research of this area worldwide (**c**). Network overlay visualisation map showing biological control agents related to the research areas for the period of 2015–2025.The bigger the size of the circle, the higher the emphasis is on that research area. The keywords reveal that South Africa focuses on the use of biological agents as natural enemies to control invasive weeds. They also show that *Solanaceae* is the most investigated family, including *Capsicum annuum*, *Solanum tuberosum*, *Solanum melongena*, *Solanum mauritianum*, and *Lycopersicon esculentum*.

The application of microbial antagonists has significant advantages, such as causing less harm than chemical pesticides, decomposing quickly, and being highly specific against the target disease and pathogen [32]. Although pathogen populations can be controlled by antagonistic microorganisms, the mode of action is not always known. Not only does the nature of the mode(s) of action determine how a pathogen population is affected by the antagonist but the characteristics of the microbial antagonist also depend on the utilized mode of action [33]. Therefore, knowledge of the mechanisms of action in the management of pests–pathogens is a key factor in achieving an efficient reduction of the pathogen in their host. Several strains cover a single mechanism, while others use a combination of them [24,34].

Various beneficial microbial antagonists have been reported for the management of plant health, but they require effective acceptance, together with the standardization of bioformulation. The present review focuses on the recent development of microbial control agents and their mechanisms of action, as well as the development of microbial pesticides and their potential applications for agricultural productivity.

2. Biodiversity of Antagonistic Microbes

Antagonism is a phenomenon whereby microorganisms colonize the rhizosphere or aerial parts of the plants without causing any harm to the plant while preventing the colonization of pathogens [35]. These microorganisms are referred to as biological control agents, biopesticides, or microbial antagonists [36]. Microbial antagonists for the control of

postharvest diseases have gained more attention in recent years as substitutes for chemical pesticides because they provide a sustainable approach to plant health management and assist in minimizing the excessive applications of toxic substances. The antagonistic microbes belong to different bacterial phyla, including *Bacillota*, *Actinomycetota*, *Pseudomonadota*, and *Bacteroidota* [1], while fungal phyla include *Ascomycota*, *Basidiomycota*, *Glomeromycota*, and *Mucoromycota* [37]. Many microbial antagonists naturally occur on the surfaces of vegetables and fruits, while others are found in closely related surroundings, such as the roots and soil [17]. They possess mechanisms that allow them to efficiently protect plants from diseases caused by plant pathogenic microbes [38]. A list of some of the microbial antagonists isolated from *Solanaceae* crops and successfully used as BCAs for the management of postharvest diseases is reported in Table 1.

Table 1. The most common bacterial and fungal biological control agents used in *Solanaceae* crops.

Biological Control Agents	Pathogens	Mode of Action	Plant Crop	Reference
Acinetobacter rhizosphaere	*Ralstonia solanacearum*	Enhances salt stress, produces plant growth hormones (auxin), and degrades organophosphorus pesticides by producing indole-3-acetic acid	Potato	[39]
Aspergillus flavus (ON146363)	*Meloidogyne incognita*	Reduces nematode propagation by producing various nematicidal secondary metabolites: gadoleic acid, oleic acid, and palmitic acid.	Potato	[40]
Azospirillum brasilense	*Pseudomonas syringae*	Phytohormone production, induced systemic resistance (ISR), and induced systemic tolerance (IST)	Tomato, potato	[41]
Azotobacter chroococcum	*Rhizoctonia solani* and *Fusarium oxysporum*	Nutrient solubilization	Tomato	[42]
Bacillus amyloliquefaciens HT	*Rhizoctonia solani*	Secretes various extracellular enzymes (protease, amylase, and cellulase)	Potato	[43]
Bacillus circulans CB7	*Dematophora necatrix*	P-solubilization and 1-aminocyclopropane-1-carboxylate deaminase activity	Tomato	[44]
Bacillus pumilus	*Fusarium oxysporum*	Activates plant defence responses under adverse conditions.	Tomato	[45]
Bacillus subtilis FJ3	*Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger*, and *Rhizopus oryzae*	Produces hydrolytic enzymes, siderophores, indole acetic acid, biofilm formation, and phosphate solubilization	Tomato	[44]
Bacillus thuriengiensis	*Fusarium oxysporum*	Produces multiple crystal proteins (δ-endotoxins)	Tomato	[45]
Bacillus velezensis DMW1	*Phytophthora sojae* and *Ralstonia solanacearum*	Antimicrobial metabolites (fengycin, iturin, and bacillomycin) demonstrated antagonistic activity in vitro and in pot experiments	Tomato	[46]
Enterobacter cloacae	*Fusarium oxysporum*	Induces systemic acquired resistance and produces antifungal compounds	Tomato, potato, and eggplant	[47]
Paenibacillus polymyxa YF	*Rhizoctonia solani*	Antimicrobial lipopeptides, including fenB, ituC, and srfAA, which are associated with surfactin, iturin, and fengycin synthesis	Potato, eggplant	[48]
Pseudomonas aeruginosa CQ-40	*Botrytis cinerea*	Solubilizes phosphorus, fixes nitrogen, and produces cellulase, protease, and ferrophilin, but it does not produce glucanase or hydrocyanic acid	Tomato	[49]
Pseudomonas putida PCL1760	*Fusarium oxysporum*	Competition for nutrients and niches	Tomato	[50,51]
Ralstonia pickettii QL-A6	*Ralstonia solanacearum*	Induces plants to up-regulate four disease-resistant defence enzymes: phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD), and superoxide dismutase (SOD)	Tomato	[52]

Table 1. *Cont.*

Biological Control Agents	Pathogens	Mode of Action	Plant Crop	Reference
Serratia marcescens	*Fusarium oxysporum*, *Ralstonia solanacearum*, and *Sclerotinia sclerotiorum*	Produces antimicrobial compounds like antibiotics (e.g., prodigiosin)	Bell peppers (*Capsicum*)	[53,54]

Acinetobacter is known for producing diffusible and volatile antifungal compounds by suppressing the disease through the mechanism of inducing plant resistance [55]. Other modes of action have been suggested as possible mechanisms behind the biological control activity of these strains, viz., siderophores and the production of HCN and lytic enzymes. *Acinetobacter* sp., as a BCA, has similar characteristics to *Pseudomonas* sp. for metabolizing various aromatic compounds like naphthalene, carbaryl, phthalate isomers, etc., as a sole source of carbon and energy [56]. The multifunctional endophytic isolate *Acinetobacter lwoffii*, Bac109, has previously been shown to be more effective than the commercial antifungal hygromycin B against various pathogenic strains like *Fusarium oxysporum*, *Bipolaris papendorfii*, *Rhizoctonia* sp., and *Phoma* sp. [57].

Bacillus species have a long history in crop growth-promoting applications under unfavorable growth conditions such as heat and drought [58]. The majority of species from the *Bacillus* genus have been shown to exhibit antagonistic activity against phytopathogenic microorganisms in agricultural crops such as tomatoes, potatoes, and peppers [59]. This genus is one of the most studied genera in terms of its production of antibiotic substances such as bacteriocins and peptide antibiotics, which are produced by *Bacillus subtilis*. *Bacillus subtilis* has been tested previously with satisfactory results to produce antifungal activity against *Botrytis cinerea*, a phytopathogenic fungus, through various modes of action, including the production of diffusible molecules (both antimicrobial molecules and siderophores); the production of hydrolytic enzymes; the production of volatile organic compounds; and competition for nutrients and space [60]. *Bacillus subtilis* has also been shown to produce the AiiA gene, which encodes for AHL-lactonase, an enzyme that breaks down N-acyl-homoserine lactones, to decrease the symptoms of diseases caused by *Erwinia carotovora* [61]. *Bacillus amyloquefaciens* secretes volatile organic compounds that inhibit *Ralstonia solanacearum* and *Penicillium digitatum* [39].

Enterobacter has been well documented as an effective BCA against *Ralstonia solanacearum* (Smith) [62], the bacterium that causes bacterial wilt in potato and eggplant. *Enterobacter cloacae* PS14 reduced potato wilt disease severity by 25%, while increasing the yield by 40% through the induction of plant systemic resistance and by suppressing the pathogen's modes of action [63]. The induction of systemic resistance can be confirmed by observing an increase in the total phenol and salicylic acid contents [64]. In tomatoes, *E. cloacae* TR1 suppresses pathogen growth by producing antifungal volatile organic compounds with 3-methylbutan-1 against *B. cinerea* [65].

Paenibacillus is known to produce many secondary metabolites, such as lipopeptide antibiotics, volatile compounds, lytic enzymes, and antifungal proteins [66]. The genus was originally included within *Bacillus* but was later reclassified as a separate genus [63].

Pseudomonas strains are commonly found in natural environments like soil and on crops such as potatoes, tomatoes, and tobacco [67]. They produce antibiotics to achieve biological control of plant diseases [68]. *Pseudomonas syringae* has been previously recorded to suppress silver scurf caused by *Helminthosporium solani* and dry rot caused by *Fusarium* spp. in potatoes [69]. Studies have shown that *Pseudomonas protegens*, *P. chlororaphis*, and *P. brassicacearum* can reduce hair root disease caused *Agrobacterium rhizogens* by up to 95% in tomatoes [70,71].

Serratia is a Gram-negative plant growth-promoting bacterium. *Serratia ureilytica* reduces the damping-off of tomato seeds caused by *Pythium cryptoirregulare*, while *Serratia marcescens* produces two known metabolites, oocydin A and biosurfactant serrawetin, which have previously shown activity against fungi and oomycetes [72].

Trichoderma is an endophytic fungus that can grow in plant tissue without causing disease through a symbiotic relationship between the host and the phytopathogens, while protecting the host and consequently improving its growth rate. *Trichoderma* controls various plant fungal and nematode diseases, including *Rhizoctonia solani*, *Fusarium oxysporum*, *Botrytis cinerea*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *Colletotrichum* spp., and *Pseudocercospora* spp. [73]. A total of 15 *Trichoderma* species are responsible for antifungal activities: *T. erinaceum*, *T. hebeiensis*, *T. taxi*, *T. bissettii*, *T. aggressivum*, *T. gamsii*, *T. reesei*, *T. citrinoviride*, *T. koningiopsis*, *T. phayaoense*, *T. longibrachiatum*, *T. viride*, *T. virens*, *T. viren*, *T. asperelloides*, and *T. asperellum* [74–76]. A total of five species play a role in nematode resistance: *T. harzianum*, *T. hamatum*, *T. atrobrunneum*, *T. lignorum*, and *T. strigosellum* [77–79]. Although studies suggest that *Trichoderma* produces antimicrobial peptides as a nematicide effect, the mechanism through which it occurs remains unclear [80]. The *Trichoderma harzianum*, T-22, species is known to have various beneficial abilities as a BCA, such as producing enzymes and metabolites that have antagonistic effects on fungi, bacteria, and nematode pathogens [81]. This species competes with pathogens for nutrients and space by colonizing the root systems and surrounding soil of plants [63,82].

3. *Solanaceae* Diseases

Bacterial soft rot is a postharvest disease that poses a serious threat to potato production worldwide [83]. Infected tubers become soft and watery and develop a foul odor. This disease is caused by two genera, *Pectobacterium* and *Dickeya*. *Pectobacterium* was previously known as *Erwinia* [84]. The contamination of potato tubers occurs during harvest, handling, and washing. The pathogen remains dormant within the plant until favorable environmental conditions, like humidity, cause a shift from latency to disease development. In South Africa, the disease has contributed to yield losses and is caused by climate change and *P. brasiliense* [85]. *Pectobacterium brasiliense* prevails at temperatures between 20 °C and 38 °C during the potato growing seasons in South Africa. The disease can be managed by spraying chemicals with copper on the wounded area to reduce the spread of bacteria to healthy plants. BCAs that have an induced systemic resistance mechanism as their mode of action can induce plant systemic resistance against soft rot by activating the salicylic acid-dependent pathway [86].

Rhizoctonia disease, also known as black scurf, is a fungal disease caused by *Rhizoctonia solani* that affects potato tubers. Although it is not destructive, it can reduce the value of potatoes because it makes potatoes hard, with black patches on their surfaces [87]. The disease can be managed using fungicide, resistant cultivars, crop rotation, and the avoidance of planting potatoes deeply [88]. Infection can occur at any time during the growing season throughout the world, including South Africa. Rhizoctonia disease was first recorded in South Africa in 1918, and currently, all 10 anastomosis groupings (AG) of *Rhizoctonia solani* associated with black scurf have been isolated in South Africa [89]. It was only recently, in 2023, that a destructive black scurf disease was recorded in Lesotho, the neighbouring country of South Africa [90]. Although most of the *Trichoderma* species have not been approved as BCA, in vitro studies have shown that *Trichoderma* species (*T. viride*, *T. herzianum*, *T. helicum*, *T. asperellum*, and *T. hamatum*) suppress the growth of *Rhizoctonia solani* by 70% [91] through different modes of action, including competition for

the substrate, antibiosis, and mycoparasitism [92]. *Trichoderma harzianum* and *T. viride* are registered as active BCAs.

Ralstonia bacterial wilt, caused by *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum*, is a devastating disease that affects tomato production worldwide [93]. *Ralstonia solanacearum* is a highly heterogeneous bacterium that can metabolize sugar alcohols and disaccharides [94]. The bacterium enters the roots through wounds made by insects, transplanting, and cultivation. Endophytic *Bacillus* species have previously been used in in vitro studies to control tomato bacterial wilt via foliar spray application. Fewer pathogens were detected on plants treated with BCA, implying that the induction of jasmonic acid, salicylic acid, and ethylene-dependent defenses was involved in the protective effects, as observed using the real-time polymerase chain reaction (PCR) method [95]. *Pseudomonas fluorescens* and *P. aeruginosa* can control bacterial wilt in tomatoes by reducing disease severity by more than 50% [96]. Evidence has shown that *Bacillus* and *Pseudomonas* species used as BCAs are promising for eradicating bacterial wilt.

Botrytis, also known as gray mold disease, is caused by *Ascomycota Botrytis cinerea*, a necrotrophic pathogenic fungus that causes substantial losses in chili peppers, tomatoes, and potatoes worldwide [97]. The disease shows various symptoms, ranging from the soft rotting of plant parts to the development of brown lesions, in addition to the production of fuzzy gray-brown mold [98]. *Botrytis cinerea* can be controlled by colonizing the pathogen with *Beauveria bassiana*, which is an antagonist that has a significant antifungal effect [77].

4. Mode of Action of Antagonistic Microbes for the Management of Plant Diseases

The perception about the mechanism of action of antagonists is a vital key factor for the effective prevention of phytopathogens from their hosts [99]. The mechanism of action related to how the biological control system occurs involves activities that include competition for nutrients and space, mycoparasitism, the secretion of antifungal antibiotics and volatile metabolites, and the induction of host resistance (Figure 2). The mechanism of action of BCA from the genus *Bacillus* is predicted by either the production of cyclic lipopeptides (LPs), such as fengycin, plipastatin, mycosubtilin, iturin, bacillomycin, and surfactin, or through the production of lytic enzymes, such as chitinase, cellulase, endoglucanase, or hemicellulase, which suppress the growth of pathogens [100]. However, in *Trichoderma*, the soluble secretome plays a significant role in modulating the mycoparasitic and antibiosis activity and in the attachment, penetration, and colonization of plant roots [101].

4.1. Competition for Nutrients and Space

The rhizosphere is a complex environment that harbors diverse microorganisms that interact with their host (host–microbe) or among themselves (microbe–microbe) [102]. The main strategy adopted by most microbial antagonists against phytopathogens is competition for nutrients such as carbohydrates, nitrogen, oxygen, amino acids, vitamins, and minerals, as well as space [25]. For a strain to be an effective BCA, it must be able to colonize plant surfaces in nutrient-deficient conditions to produce a beneficial effect. Therefore, for greater effectiveness, microbial antagonists can rapidly colonize the fruit wounds prior to colonization by the pathogen [103] by assimilating the carbon sources for survival and proliferation, thereby depleting carbohydrates and starving the competing pathogens [104]. Understanding the mechanism used by BCAs to compete and interact with other microbes within the rhizosphere is crucial for their effective colonization. This intrinsic mechanism has been described in several biological control studies on antagonists such as *Serratia plymuthica* and *Pseudomonas agglomerans* [105]. A saprophytic fungus,

Trichoderma spp., which is known for its fast mycelial growth, controls the invasion of pathogenic fungi in the root of a plant by rapidly absorbing the nutrients required for the growth of the pathogen fungi, resulting in nutrient deficiency and inhibiting the growth and reproduction of the pathogen fungi [106]. The growth rate of *Trichoderma* is much faster than that of *B. cinerea*, *Fusarium solani*, and other pathogenic fungi [107]. Consequently, it can capture water and nutrients, occupy space, and consume oxygen faster to weaken and exclude pathogens in the same habitat [73].

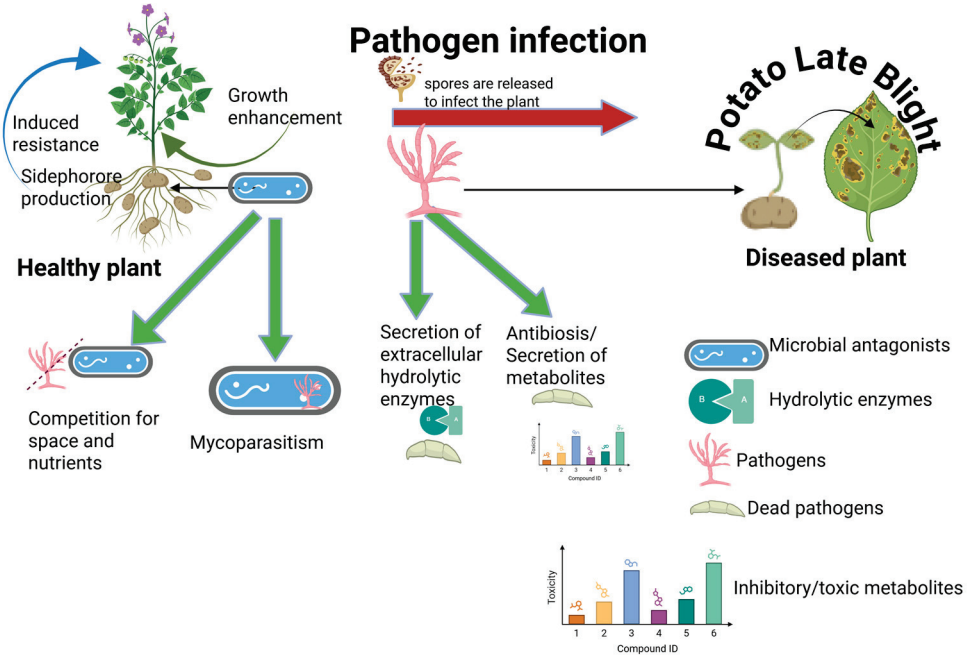


Figure 2. Key mechanisms of action involved in the biological control of potato late blight disease by microbial antagonists. The mechanism of action of biocontrol agents (BCAs) is an intricate process that consists of microbial antagonists, pathogens, and the host plant. BCAs compete with the pathogen for nutrients and space, produce various types of phytohormones and siderophores, and cause nitrogen fixation and solubilization through induced systemic resistance and mycoparasitism (where one fungus parasitizes another), resulting in improved plant growth and, hence, the control of plant disease. However, in the absence of BCAs, a pathogenic fungus can infect a potato plant, causing potato late blight disease, resulting in the production of toxic metabolites. Compound ID represents toxic metabolites: 1-atropine, 2-hyoscyamine, 3-scopolamine, 4-pyrrolic alkaloids, and 5-pyrrodine. This figure was created using Biorender.com.

4.2. Mycoparasitism

Mycoparasitism is a common form of antagonism that involves the direct physical contact of the mycoparasite with the host hyphae. The mycoparasite often coils around the hyphae, penetrates the host, absorbs nutrients, and, finally, disintegrates the cells of the host to produce extracellular cell wall lytic enzymes. [56]. The produced extracellular lytic enzymes include cellulases, lipases, proteases, chitinases, and glucanases [108]. Chitinases are glycosyl hydrolases that directly degrade chitin to low-molecular weight chitooligomers. Chitin is the second most abundant polysaccharide after cellulose [109]. *Trichoderma* spp. can parasitize *Sclerotinia sclerotiorum*, *Rhizoctonia*, *Pythium*, *Peronospora*, and *Phytophthora* by directly invading the mycelium, causing the pathogen cells to expand, deform, and shorten, while shrinking the protoplasm and breaking the cell wall [110].

4.3. Antibiosis

Antibiosis refers to the interaction between two organisms, whereby the growth and activity of one organism are inhibited by the other organism through the production of chemical compounds [111]. *Streptomyces* sp. produces antibiotics, enzymes, and other antimicrobial compounds that directly suppress pathogens [67]. *Bacillus* sp. also suppresses the growth of pathogens via an antibiosis mechanism [112]. Similarly, *Trichoderma* sp. inhibits the growth of the nematode *Meloidogyne incognita* by producing secondary metabolites, including terpenes, polyketones [113], trichomycin, gliotoxin, viridin, antibacterial peptide [114], chitinase, butyrolactones, and some volatile substances (esters, terpenoids, furans, aldehydes, alkanes, olefins, and heterocyclic compounds [115]. Other bacterial genera that suppress postharvest microbial pathogen growth by producing antibiotics include *Burkholderia*, *Pseudomonas*, *Enterobacter*, and *Lysobacter* [116].

4.4. Induced Systemic Resistance

Induced systemic resistance (ISR) is a mechanism by which selected plant growth–promoting bacteria and fungi in the rhizosphere prime the whole plant body for enhanced defense against various pathogens and insects [117]. Beneficial microorganisms need to recognize the presence of pathogens to avoid forming symbiotic relationships [80]. Beneficial bacteria, such as *Pseudomonas* spp. and *Bacillus* spp., stimulate defense responses and help plants obtain broad-spectrum disease resistance [83], while beneficial fungi, such as arbuscular mycorrhizal fungi (AMFs) and *Trichoderma* spp., induce systemic resistance and defense against *Phytophthora parasitica* in tomatoes and *Botrytis cinerea*, respectively [74].

5. Difficulties Encountered During the Commercialization of Biological Control Agents

The commercialization of BCAs begins in the field with identifying the target crop and the pathogen, understanding current disease-control strategies, isolating potential microbial strains [118], and taxonomic classification using 16S rRNA gene sequencing in the lab [119]. The screening process is a crucial, time-consuming process that is followed by bioassay tests to determine its efficacy on a laboratory scale in a controlled environment. For example, to screen BCAs to address the postharvest diseases, microbes must be screened from the surface of the fruit/vegetable, instead of screening for microbes that compete for soil colonization. The efficacy of a potential BCA must be tested later at different geographical locations, under different climatic conditions, and on different crops against a range of pathogens to evaluate their potential for broad-spectrum activity. However, the consistency of the BCAs is not maintained in an uncontrolled environment because of their modes of action [120].

Several commercial BCAs have been jointly developed by researchers working with commercial companies (Table 2) [121]. As measured by their widespread acceptance and use, the potential commercial biological control products have produced low success rates because of inconsistent performance under commercial conditions. The efficacy of these products must be similar to that achieved by agrochemicals, which is in the range of 98–100% disease control in the field [122]. However, a greater success rate has been observed under greenhouse conditions, contributing to a higher number of biological control products being produced. To date, 390 microbial biological control agents (MBCAs) have been approved by the Environmental Protection Agency (EPA) [104]. Similarly to synthetic pesticides, BCAs are subjected to risk evaluations according to the European Regulation (EC) No. 1107/2009 procedures for plant protection product marketing before they can be licensed [102].

Table 2. List of approved commercially available biological control agents and their target pathogens.

BCA	Commercial Name	Target Pathogens/Disease
Bacillus amyloliquefaciens strain MBI 600	SERIFEL®	*Botrytis cinerea*, *Sclerotinia* spp.
Bacillus amyloliquefaciens strain FZB24	TAEAGRO®	Powdery mildew diseases, *Botrytis* sp.
Bacillus amyloliquefaciens subsp. *plantarum* strain D747	AMYLO-X®	*Botrytis cinerea*, *Monilinia* spp., *Sclerotinia* spp.
Bacillus pumilus strain QST 2808	SONATA®	Powdery mildew diseases
Pseudomonas chlororaphis strain MA342	PRORADIX®	*Rhizoctonia* spp., *Helmintosporium solani*, *Fusarium* spp.
Pseudomonas chlororaphis strain 63-28	AtEze®	*Pythium* spp., *Rhizoctonia solani*, and *Fusarium oxysporum*
Streptomyces lydicus strain WYEC 108	ACTINOVATE® AG	*Rhizoctonia* spp., *Verticillium* spp., *Phytophthora* spp., *Fusarium* spp., *Alternaria* spp., *Botrytis* spp.

6. Future Directions and Challenges

Utilizing microbial antagonists to control postharvest diseases is a viable strategy for reducing the need for agrochemicals and mitigating the detrimental effects of phytopathogens on crop productivity. However, there are still several challenges that need to be addressed to fully utilize these BCAs effectively and sustainably. The challenges are as follows: BCAs might not be as effective as well-established pesticides, environmental factors such as temperature can be a factor because some of the bacterial strains function better at optimal temperatures, and the cost of manufacturing might be too high to allow for profitability, making BCAs inaccessible to most farmers. Most developing countries continue to produce and use BCAs without registration to avoid the high registration costs, while some companies register BCAs as biofertilizer, not as BCAs, for general use in all agricultural crops.

Since BCAs impact the natural environment, one must preferably predict any possible risks that could result from their implementation because their mechanisms of action for suppressing pathogens are not always known. Therefore, a comprehensive analysis of their advantages and hazards should be conducted to give stakeholders the knowledge they need for effective, secure, and long-term pest control and production and to eliminate the fear of the unknown. Knowing the molecular mechanisms behind these interactions is a crucial part of understanding how the plant host reacts to this interaction, as well as how phytopathogens react to BCAs, or vice versa. Furthermore, the method by which plants distinguish between beneficial and harmful bacteria is still unknown. However, it has been proposed that plants can adjust their microbiomes by releasing certain nutrients to increase competitiveness. Nevertheless, with all the proper isolation, screening, and field trials needed to scale up the potential microbes from the lab to the agricultural field, there is always a delay in the regulatory approval, registration process, and marketing of products. However, diverse effective BCAs are available commercially. By addressing most of these challenges, potential BCAs might contribute to the sustainability and effectiveness of disease management while promoting a more environmentally friendly agricultural system.

7. Concluding Remarks

Microbial antagonists have long-term potential in improving sustainable agriculture, with more than 440 currently available species that act as control agents for various pests and pathogens, from the most widely researched rhizobacteria, *Bacillus*, to *Paenibacillus*, which is the least researched after *Serrati* because it was reclassified as a separate genus. These microbial antagonists are involved in a variety of mechanisms, including competition for nutrients and space, antibiosis, and the induction of systemic resistance, that inhibit some plant diseases and, hence, reduce the need for agrochemical pesticides. By unraveling these mechanisms, researchers can develop targeted strategies for enhancing

the colonization efficiency and overall effectiveness of biological control bacteria, leading to more sustainability and resilience. Solanaceae crops, such as *Solanum tuberosum*, *Solanum lycopersicum*, *Capsicum annuum*, and *Solanum melongena*, are globally significant and are known to be susceptible to a wide range of bacterial and fungal pathogens, resulting in substantial economic losses. This susceptibility makes them an ideal target for researchers to develop BCAs to reduce reliance on fungicides and pesticides to fight pathogens and to ensure food security and economic stability. Although microbial formulations for commercialized BCAs, such as liquid and solid formulations, exist, a major bridge between fermentation and field application still exists because of factors such as production at a large scale, stabilizing the BCAs during shelf life, the handling and use of the product, and ensuring the viability efficacy at the target site. Nevertheless, we can positively conclude that the prospects of BCAs are promising and that they will benefit the environment in controlling crop diseases.

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Abbreviations

The following abbreviations are used in this manuscript:

BCA	Biological control agents
GDP	Gross domestic product
LP	Lipopeptides
EPA	Environmental Protection Agency
ER	European Regulation

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Review

Improving Plant Performance Through Microbiome Manipulation: The Potential Role of Current Bioengineering Approaches

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Abstract: In the recent past, microbiome manipulation has emerged as a promising approach to improve plant growth performance by exploring the deep insight of plant–microbe interactions. The exploration of a plant microbiome either present on an ectosphere or endosphere can provide a far better understanding about the potential application of plant-associated microbes for the improvement of plant growth, protection from pathogen invasion, and tolerance to environmental stresses of a diverse nature. In this context, next-generation sequencing methods, omics approaches, and synthetic biology have made significant progress in plant microbiome research and are being frequently used to explore the intriguing role of plant-associated microorganisms. Despite the successfulness of conventional approaches, the incorporation of CRISPR/Cas9, RNA interference technology, rhizosphere engineering, microbiome engineering, and other manipulation techniques appear to be a promising approach to enhancing plant performance, and tolerance against biotic and abiotic stress factors. The present review presents the significance of plant microbe interaction, vital functional aspects, collaborative action, potential constraints, and finally the latest developments in bioengineering approaches destined for microbiome modulation with an objective to improve the performance of a host plant challenged with environmental stressors.

Keywords: rhizospheric engineering; RNAi; microbiome engineering; microbial manipulation; plant–microbe interactions

1. Introduction

Currently, agricultural productivity is facing challenges to meet the escalating food demand of a continuously rising global population not only because of declining cultivable land areas, but also due to unfavorable climate change. According to an estimate, agricultural productivity needs to be enhanced by 60% by the year 2050 [1]. Noteworthy is that climate change and a limited availability of natural resources such as agricultural land and water availability limits agricultural productivity [2]. During the past two decades, a substantial progress in agricultural practices has been marked; nevertheless, abiotic environmental stressors such as drought, salinity, flood, nutrient deficiency, soil degradation, indiscriminate application of hazardous agrochemicals, and biotic stress factors including resistance resurgence in pathogens have resulted in compromised crop productivity, suggesting the development of environmentally sound strategies to fulfil the food demand.

Plants and microbes are considered to be continuously co-evolved by forming a complex ecosystem, thereby considerably influencing the growth as well as the survival of each other. The concept of "holobiont" has come into existence to reveal the interaction between the host and associated microorganisms in a given environmental complex with plausible implications in biology, ecology, and the evolutionary process of the host plant. Furthermore, the "holobiont" framework comprising of the host and associated microorganisms is suggested to evolve synergistically for mutual cross-talk and co-signaling [3,4]. The diverse microbiome existing in the rhizosphere, phyllosphere, and endophytically in different plant parts with the characteristics ability to augment host plant growth are referred to as "plant-growth promoting microorganisms" (PGPMs) [5,6]. The PGPMs comprising of different bacteria, fungi, etc. are acknowledged to enhance agricultural productivity [1,7–9].

Recently, microbiome manipulation has been carried out to improve agricultural productivity or maintain the plant health via different processes such as tillage practices, synthetic microbial consortia, microbial inoculation, rhizosphere engineering, and microbiome engineering [10,11] (Figure 1). Although these approaches are dependent upon various factors such as the host plant, associated microbial communities, soil, and other environmental factors, manipulation of these factors directly or indirectly influence plant growth or developments.

Therefore, there is a need to explore various aspects of plant microbe research, microbiome functioning, and how the microbial communities influence plant growth [12,13]. This review aims to provide an overview of the latest developments in microbiome manipulation. Additionally, various aspects of plant–microbe interactions, traditional approaches of microbiome manipulation, and how plant performance can be improved using the latest emerging approaches such as CRISPER/Cas9, RNAi, or microbiome engineering, etc. are discussed.

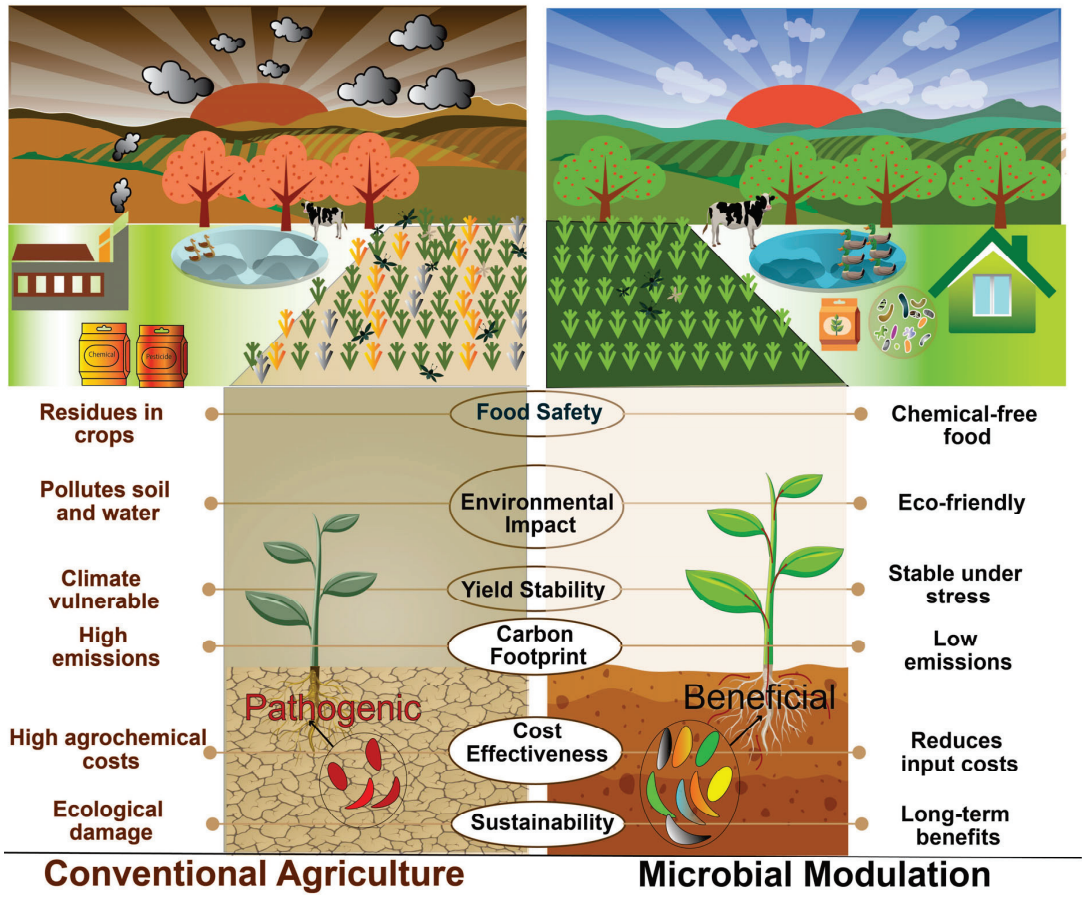

Figure 1. An overview of microbial modulation or manipulation techniques in sustainable agriculture practices. This figure illustrates the contrasting ecological impacts of microbial manipulation/modulation versus conventional cropping on various environmental components such as soil, air, and water. Microbial modulation promotes eco-friendly practices, enhances soil health, and reduces pollution in air and water. It supports long-term sustainability, stable yields under stress, and chemical-free food production. In contrast, conventional cropping contributes to soil degradation, water contamination, and air pollution, with high emissions, pesticide residues, and vulnerability to climate change, while relying on costly and harmful agrochemical inputs.

During the review process, we carried out a bibliometric data analysis to evaluate the significance of topic microbiome manipulation in improving plant performance. We retrieved 68,401 articles published between 2000 and 2025, from the PubMed database by searching for the keywords "Microbiome Manipulation" OR "Microbial Modulation" And "Plant health" OR "Plant performance" OR "Microbiome engineering" OR "Rhizosphere engineering" OR "CRISPER/Cas" or "RNAi" OR "Bioengineering approaches" OR "Holobionts" OR "omics" OR "crop rotation" OR "plant—microbe interactions" OR "tillage intensity" OR "Conventional Agriculture " OR "Biotic stress" OR "abiotic stress " (data accessed on 15 February 2025). Out of 15,462 keywords, 277 were selected with a cutoff of 15 occurrences and we downloaded 10,000 papers in a tab-delimited format and created bibliometric analysis (Figure 2) using VOS viewer processing software (v1.6.9). The different colors present in the network show linked terms; however, the size of the labels showing the number of publications and the distances present between two nodes represent the degree through which they are associated.

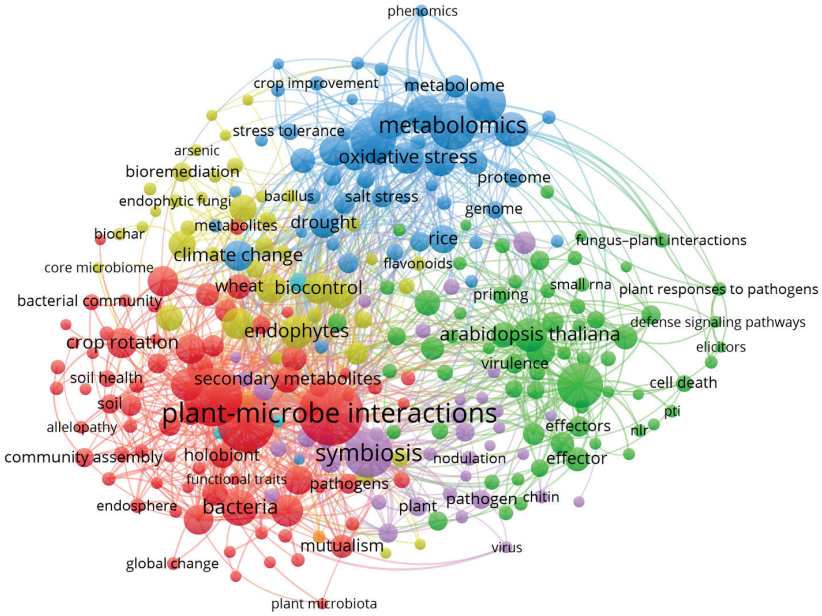

Figure 2. Network visualization map displaying the key term present in the title and abstract of the published article related to the plant–microbe interactions in abiotic stress management. Nodes of the same color show a cluster of the interconnected phrases; however, the circle size indicates the number of publications, with the larger size indicating a large number of publications. The network has been created using the software VOS viewer processing software (v1.6.9.).

2. Plant–Microbes Interactions and Their Significance

Plants interact with a plethora of microbial species, in which some of them directly or indirectly play a significant role in plant growth and developments [13]. The plant rhizosphere is considered as a hotspot of microbial communities and is defined as a thin layer of soil that surrounds and is influenced by plant roots. The plant root secretes an array of exudates constituted of amino acids, flavonoids, lipids, organic acids, and other secondary metabolites, and these biochemical components act as an energy source or signaling molecules for the microbial species [14–17]. The amount and the nature of exudates varies with internal or external surrounding factors such as host genotype, seasons, temperature, nature of the soil, etc., which also influence the composition of host-associated microbial communities [18,19]. For example, during the seedling stages, the nature and amount of sugar molecules is different than at the harvest stages; similarly, draught or other stress factors also influence the amount and nature of exudates, which ultimately affect the microbial community structures [20].

Each of the plant organs such as the root, stem, leaves, flowers, etc. have some specific microbial composition which may vary with changing environmental conditions, host genotypes, plant developmental stages, seasons, or stress factors [13]. The microbial communities structure of the plants are generally dominated by the bacterial phyla Proteobacteria, Bacteroidetes, Acidobacteria, and Actinobacteria [21–23] and by the fungal phyla Ascomycota and Basidiomycota. In the recent past, various authors studied the microbial communities structure of different plant species. For instance, Fu et al. [24] reported Proteobacteria and Actinobacteria as the most prominent bacterial phyla in the semiarid alpine grassland. Nan et al. [25] reported the dominance of bacterial phyla Actinobacteria and Proteobacteria in the rhizosphere of three species of Stipa. Xu et al. [26] in the global study of citrus rhizosphere also reported Proteobacteria, Actinobacteria, and some other bacterial group like Acidobacteria and Bacteroidetes as the prominent one, while Ascomycota and Basidiomycota were the dominant fungal phyla.

It has been observed that the endosphere contains lesser microbial species than the ectosphere or rhizospheric soil. For example, an abundance of bacterial population in the rhizospheric soil was reported in the range of 10^6–10^9, while only 10^4–10^8 CFU (colony forming unit) per gram of root tissues was observed in the plant endosphere [27–29]. In studies of the plant endosphere, a dominance of Proteobacteria was also reported followed by Actinobacteria. Singh et al. [30] also reported an abundance of phyla Firmicutes and Proteobacteria as endophytic in the *Momordica charantia* L. Goulart et al. [31] observed a higher abundance of Proteobacteria in the vegetative and reproductive stage of *Passiflora incarnata*. Other bacterial phyla that were dominant in the vegetative growth were Firmicutes followed by Actinobacteria and Bacteroidetes, while Firmicutes (28.5%) was the second dominant group in the reproductive stages.

3. Functional Aspect of Plant Microbiomes

The host plants are considered to offer a habitat for a multitude of microbial communities. The intimate association of microorganisms with plants has been acknowledged to facilitate plant growth [32]. The extensive investigation on the microbiome associated with plants has helped in understanding the intricate communications [33]. The employment of a beneficial microbial population has demonstrated a characteristic role in augmenting crop productivity under changing climatic conditions, diversified stress factors, and a deteriorating soil environment [34–36].

Stresses either of biotic and abiotic negatively influence crop productivity worldwide. Noteworthy is that abiotic stress factors have emerged as a major constraint that limits agricultural productivity. The microbial association with the host plant helps alleviate the repercussions of abiotic stressors through the biosynthesis of polymeric substances including exopolysaccharides serving as a protective barrier in the root zone, in addition to the release of osmolytes, including sugar and amino acids as illustrated in the strains of *Acinetobacter*, *Pseudomonas*, and *Pantoea* recovered from drought-resistant and salt-tolerant plants [37]. Additionally, phytohormones and volatiles released by host-associated microbiome triggers induced systemic tolerance (IST). The microbiological biosynthesis of enzyme ACC deaminase lowers the ethylene level. For instance, *Achromobacter piechaudii* ARV8 equipped with the ability to produce ACC deaminase has been suggested to provide tolerance against drought in *Capsicum annuum* L. and *Solanum lycopersicum* L. [38]. Salinity has been envisaged as an important stressor limiting crop productivity substantially. Leveraging salt-tolerant microorganisms could be a sustainable option to mitigate the challenges of agricultural productivity. A recent investigation by Ning et al. [39] explored the feasibility of leveraging halotolerant bacterial strain *Enterobacter asburiae* D2, endowed with a multitude of growth promotion attributes, to elevate the challenges of salinity stress in *Oryza sativa* L. Khumairah et al. [40] and Sarker et al. [41] reported on halotolerant rhizobacteria, which after inoculation significantly improved the salinity tolerance and enhanced plant growth [40,41].

PGPMs and their products such as biofilm, exopolysaccharides, and biosurfactants are frequently utilized to mitigate the challenges of heavy metal accumulation in the soil. In addition to mitigation processes of heavy metals, PGPMs also help in growth promotion of plants [42]. For example, treatment with *Klebsiella* sp. M2 led to an increase in dry weight ranging from 14.3 to 35.9% and the release of metabolic constituents responsible for growth promotion in *Triticum aestivum* L. Heavy metal–tolerant microbial strains of endophytic nature and those residing in the rhizospheric zone can be exploited for the amelioration of heavy metal toxicity in crop plants. Previous authors reported various different bacterial

species such as *Enterobacter*, *Psudomonas*, *Bacillus*, and *Klebsiella* having the capability to accumulate excess heavy metals [43].

A microbiome can be harnessed for encountering the rising threats of crop damage caused by different phytopathogens. The microbial species having the ability to protect the plants from pathogen invasion or plant diseases are termed as "microbial biocontrol agents." The microbial biocontrol agents inhibit the phytopathogen growth by secreting antimicrobial metabolites, antibiotics, siderophores, volatile bioactive compounds, etc. or by host immune response or by inducing systemic response [44]. The phenomenon of induced systemic response (ISR) directs the expression of key genes related to defence, such as phenylalanine ammonia lyase (PAL), peroxidase, chitinase, etc. [1]. Also, the release of secondary metabolic products like quorum sensing biomolecules that aggravate the event of ISR. Furthermore, different microbial species can exert various direct as well as indirect effects involving key strategies like enzymes secretion, which causes cell wall degradation, and volatile components restricting the metabolic activities with the resultant inhibition of phytopathogens multiplication [33].

Quorum sensing (QS) is a type of microbial communication mechanism in which bacteria communicate and work together through various chemical cues referred to as "auto-inducers" to govern populations and sustain the equilibrium of a certain process. Additionally, QS modifies the gene expression patterns in response to cell density, which leads to the modification or activation of various transcriptional factors in bacteria [45]. QS also showed direct involvement in the regulation of various functions such as biofilm formation and colonization within the bacterial populations, which plays a significant role in plant growth promotion even under stress conditions [46]. However, the QS molecules like N-acyl homoserine lactones (AHLs) induce the systemic resistance in plants, making them resistant against the pathogens [47].

However, the full potential of a microbiome with reference to sustainable agriculture under diverse climatic conditions has to be explored. The search for newer microbial species, their application as soil or plant inoculants, and the creation of synthetic microbial consortia to control the phytopathogen growth needs to be studied more extensively. The following section deals with the collaborative action of a plant microbiome and strategies being employed for microbiome manipulation using conventional and modern bioengineering approaches.

It is well established that plant-associated microbial species play a significant role in improving plant performance via enhancing plant growth or controlling phytopathogen growth. However, in natural and engineered ecosystems, millions of microbial species reside or co-exist and each possess specific functions and metabolic capabilities [9]. These microbial species perform some specific community's level performance that contribute a significant role in maintaining plant stability and health during normal or stress conditions [12]. In general, both an energy source and nutrient requirements are fundamental needs for the survival of microorganisms. Although within the microbial community the requirement of an energy resource and the nutrient supplements depends upon the reciprocal exchange of metabolites within the different microorganisms present in the community [12].

4. Dysbiosis in Plant Microbiomes

Dysbiosis signifies an alteration within a microbial community that negatively affects the health of particular plants. Some environmental factors like stress, nutrient content in the soil, and different types of pathogens can throw off the balance within microbes [48]. For example, the increased presence of pathogenic microorganisms within the mycorrhizo-

sphere may suppress the recruitment of good mycorrhizae, thereby reducing plant health and productivity [49]. In addition, certain plants may produce allelochemicals that modify the makeup of their associated microbiomes, resulting in poor interactions with plants, which may lower plant performance [50]. The consequences of dysbiosis are not limited to individual organisms but rather whole ecosystems. The alterations of the diversity of microbes have consequent effects on the microbially mediated processes of nutrient cycling, the health of soils, and the composition of plant communities.

5. Approaches for Microbial Manipulation

In the recent past, a number of studies have suggested different approaches for the manipulation of the microbiome in order to improve plant fitness and agricultural productivity so as to meet the rising food demand for an incessantly growing human population. Some of the commonly used methods such as crop rotation, organic fertilizer input, tillage intensity, and microbiome reorganization are presented in the following sections.

5.1. Crop Rotation

Crop rotation is a common traditional approach practiced to maintain soil productivity and modulate the native microflora of the agricultural soil. It has been observed that repetitive cropping of the same crop can lead to a loss of moisture content, organic matter, and native microbiota [51]. Therefore, maintaining the nutrient status and composition of native microbiota rotation in the cropping system has been preferred for a long time. In a recent study, Yang et al. [52] acquired six years of data and reported a significance of diversified crop rotations and observed that an addition of sweet potato and legumes plants viz. peanut and in the traditional wheat–maize rotation significantly enhanced the soil microbial diversity and agricultural output up to 38%. Additionally, it reduced the emission of N_20 by 39% and improved the greenhouse gas balance by 88% in the North China plain. Similar types of observations like growing maize after the cultivation of rice can help with nutrient acquisition and the assembly of beneficial microbial communities studied and reported by various authors in the previous studies [53,54].

5.2. Organic Fertilization

Currently, a huge amount of chemical fertilizers and pesticides are being used to enhance agricultural productivity, but their continuous utilization adversely affects the texture and productivity of plants, native microflora, and human health [6]. Therefore, organic amendments, or bio-organic fertilization, are now being used as efficient methods for maintaining soil productivity or native microflora [55,56].

Soil health and the functioning of an ecosystem largely dependent upon the interactions between the soil organic matter and the native microflora. The presence of a higher content of organic matter positively modulates the growth and metabolism of microbes and also shapes the microbial community structure and diversity [56,57]. The presence of a diverse microbial community not only helps in maintaining soil fertility but also helps in degrading complex environmental contaminants. Delgado-Baquerizo et al. [57] reported that the presence of a diverse microbial community modulates the breakdown of litter and other complex organic matter. Similarly, Sun et al. [58] reported that nutrient-rich leaf litter is more easily decomposed than lignin-rich woody litter, which can constrain microbial growth if not adequately supplied [58].

In conventional practices, nutrient-rich agricultural residues, organic manures, biocomposts, or vermicompost have been used to improve and maintain the soil texture or

productivity [59]. Previous authors reported the use of bio-organic fertilization in improving plant growth [60–63]. For example, Lahbouki et al. [64] used horse manure, and Arbuscular Mycorrhizal Fungi (AMF) to amend the soil and enhance tomato productivity. Li et al. [65] used different bio-organic fertilizers to evaluate the impact on *Dendrocalamus farinosus* growth and found that the utilization of bioorganic fertilizers enhances the growth via restructuring the soil microbiome and metabolome. Wang et al. [66] reported the impact of bio-organic fertilizers on the yield enhancement of pear, which also shapes the rhizospheric microbiome. Xu et al. [60] reported that organic fertilizers like black soldier fly frass derived from different organic wastes can significantly benefit plant growth by improving soil fertility and microbial diversity. Similarly, a combination of sheep manure and chemical fertilizers showed a beneficial role in plant growth promotion [61]. Furthermore, the application of organic manure in Qingke cultivation has been found to influence the rhizosphere microbiota composition and plant growth [62]. More field studies, however, are required to translate the advantageous actions of the microbiome for augmenting the productivity of a plant challenged with diverse abiotic and biotic factors.

5.3. Tillage Intensity

Tillage intensity refers to the degree and frequency of soil disturbance caused by agricultural practices. It ranges from intensive to no tillage intensity, which involves significant soil disruption, to minimal or no disturbance in soil. In tillage practices, the relocation of crop residues has been carried out, which affects and redistributes moisture, the available carbon, and oxygen availability to the soil [67,68]. Currently, different types of tillage intensity such as conventional tillage, reduced tillage, or no tillage has been practiced that significantly affect soil structure, organic matter distribution, and the soil microbiome [69–71]. In previous studies, authors reported on different tillage practices and their impact on maintaining soil or plant health. Dewi et al. [69] reported on long-term no-tillage and integrated the use of biochar to improve soybean production. Hu et al. [70] observed that a reduced tillage system improved carbon, nitrogen, and phosphorous in the soil system.

5.4. Reconstructing the Existing Microbiome by Microbial Inoculation

In sustainable agricultural practices, the screening and characterization of a specific microbial strain is one promising approach [72]. Advancement in the latest next-generation sequencing technology, metagenomics, metabolomics, and transcriptomics study enhanced the understanding of microbial community structure and their functioning. These studies also help in characterizing the microbial strains, having better suitability and capability to use as soil or plant inoculants to enhance agricultural productivity [73]. Although for successful inoculants, the strains must have the capability to survive under field conditions and have the potential to be outcompeted by the native microbiome after the introduction [74]. The transition of microbial strains or consortia from the laboratory to the field condition is one of the most challenging tasks. It has been observed that transition sometimes diminishes the efficacy and survivability of microbial strains [75].

In contrast, in recent times, inoculation of two or more than two microbial species consortia either of the same genera or different genera, such as bacteria–bacteria, bacteria–fungi, bacteria–fungi–yeast, have been formulated to create a diverse functional genetic pool, having the potential to act multidimensionally against environmental challenges or stresses [76]. The synthetic consortia of different microbial strains shows potential to resist against the multiple constraints, where the single strain only has limited options. For instance, under low-phosphorus condition, inoculation of phosphate-solubilizing

bacteria along with a synthetic consortia of multiple strains showed a superior effect in the crop growth [74]. These consortia effectively provide sufficient solubilized phosphorus, demonstrating their potential to enhance plant growth in nutrient-deficient soils. Wang et al. [77] identified and characterized 23 strains to create a potential synthetic consortia of different strains to promote rice growth. Manjunath et al. [78] reported two different synthetic consortia, *Pseudomonas putida* P7 + *Paenibacillus favisporus* B30 and *Pseudomonas putida* P45 + *Bacillus amyloliquefaciens* B17, to successfully enhance the growth of maize plants by 27.78 and 23.21%. Similarly, Olanrewaju and Babalola [79] used different microbial strains singly or in combination to evaluate their potential on maize seed growth and found that a mixture of consortia was more potent than a single inoculation. Similarly, Yin et al. [80] created 10 synthetic consortia of 14 different bacterial strains in which nine syncoms showed an antagonistic role against the wheat pathogen *Rhizoctonia solani* AG8.

6. Engineering Approach to Improve Plant Performance

Despite the successfulness of conventional approaches suggested for microbiome modulation, the introduction of current bioengineering approaches is considered to have promising potential in improving plant fitness under stressed conditions. Some methods of microbiome manipulation including rhizosphere engineering, deliberated alteration in microbiome characteristics, CRISPR/Cas9, and RNA interference with a profound effect in offering tolerance to host plants under environmental stress are presented in the next section.

6.1. Rhizosphere Engineering

Rhizosphere engineering is an emerging approach through which plant performance has been improved following some key strategies like modification in the root architecture to enhance the root absorption area, modifications in the amount and nature of root exudates to attract some specific microorganisms, plant engineering to overexpress or knockout the genes to attract specific microbial taxa, and soil amendments to promote the growth of some specific plant growth–promoting bacteria [81–83].

In previous studies, authors reported on various approaches of rhizosphere engineering. In most of the studies, during root engineering, the primary focus is to knockout the genes that are responsible to check the growth and developments of the root system [84,85] and the functional aspect of the root system can be modulated by modifying the gene expression pattern [86]. Modification in the root architecture is another strategy of rhizosphere engineering to improve the plant performance. In this approach, the architecture of the root has been modified in such a way that the root can hold or absorb a better amount of water and nutrients [84,87,88].

The composition, amount, and the nature of the root exudates affect interactions patterns between the host and microbes and are influenced by the plant development stages, plant genotypes, soil conditions, or the surrounding environmental factors [89]. These root exudates act as chemo attractants or chemo repellents for the microbial species. Modifications in the composition or specific molecules in the root exudates are one of the strategies of rhizosphere engineering, through which specific microbial species enriched in the plant rhizosphere enhance and modulate microbial growth [90].

Inoculation of the soil with beneficial microbial strains is used to improve plant growth and productivity [91,92]. Although environmental conditions and plant species are vital for the success of rhizosphere engineering, which is based on selection and adaptation of microbial strains, recent improvements in high-throughput sequencing and metagenomics have supported the characterization and recognition of beneficial microbial strains from different environmental conditions [91]. For example, unique strains of *Bacillus* and *Paenibacillus* were recruited from

the rhizosphere of drought-tolerant plants and these were later utilized to improve drought resistance in new crops [92]. Likewise, another suggested track to recover plant performance is the insertion of specific genes into microbial genomes, to progress the microbial performance. For example, *Pseudomonas fluorescens* was obtained to produce the plant hormone indole-3-acetic acid (IAA), subsequent in progressed root growth and nutrient exploitation in wheat, which demonstrated the microbial functions [93].

6.2. Microbiome Engineering

Microbiome engineering is receiving accelerated attention in view of improving crop productivity and encountering the hurdles of climate change [94–97]. Although different plant parts are known to harbor specific microbial communities and are endowed with the potential to influence the host biology [98], during the process of microbiome engineering, the microbial communities structure of the plant has been modified or optimized (Figure 3). This involves various processes viz. modification of indigenous microbial strains with the help of genetic engineering approaches like CRISPER/Cas9, RNAi interference, etc.; transplantation of beneficial microbiome from the healthy to the target sites; or the development or inoculation of syncoms. However, the primary goal of microbiome engineering is to improve the functioning of microbes in such a way that shows an improved stress tolerance potential, improved nutrients utilization patterns, etc. [99,100].

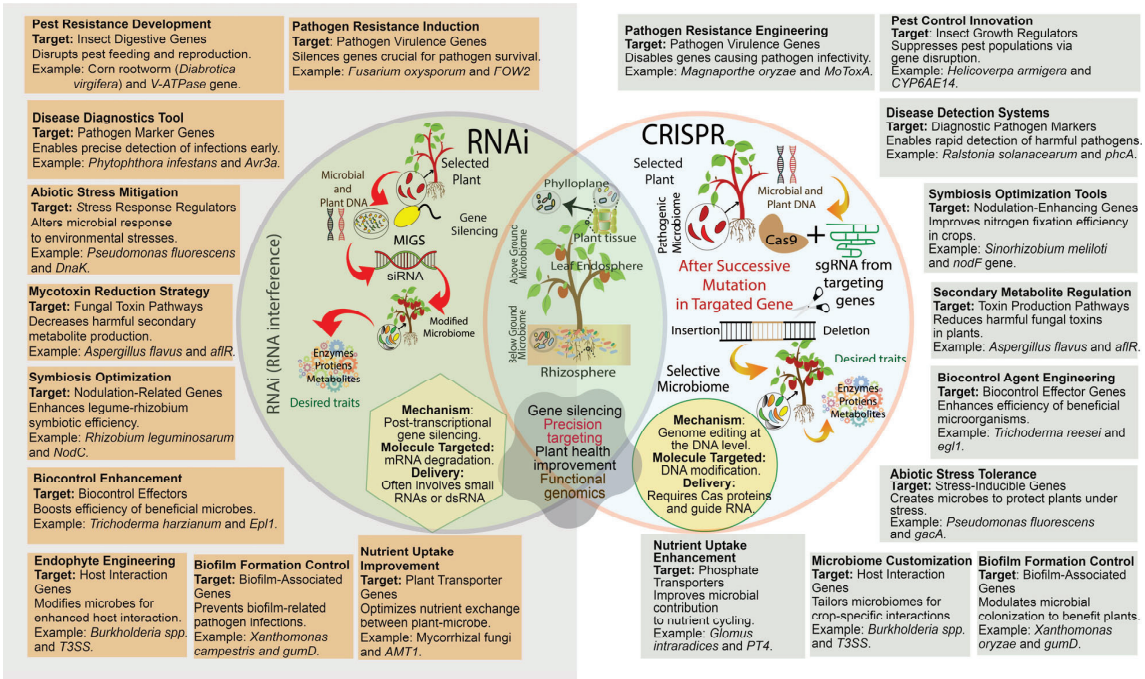

Figure 3. The interplay between RNAi and CRISPR technologies in plant–microbe interactions, emphasizing their distinct and overlapping roles in microbiome engineering. RNAi (**Left Circle**) is a post-transcriptional gene-silencing mechanism targeting mRNA degradation, widely used for pest control (e.g., silencing digestive genes in *Corn rootworm*) and mycotoxin reduction (e.g., *Aspergillus flavus* toxin pathway). CRISPR (**Right Circle**) is a genome-editing tool targeting DNA, and facilitates precise modifications for pathogen resistance (e.g., editing *Magnaporthe oryzae* virulence genes) and nutrient uptake enhancement (e.g., improving phosphate transport in *Glomus intraradices*). The intersection of RNAi and CRISPR demonstrates shared applications such as gene regulation, precision targeting, and improving plant health. Applications span pathogen resistance, symbiosis optimization, biocontrol enhancement, and abiotic stress tolerance, showcasing the synergistic potential of these tools in advancing sustainable agriculture and functional genomics.

The identification of genes or alleles responsible for the pathogenicity, synergistic, or antagonistic effect is a prime concern in microbiome engineering and their modifications either by enhancing protein expression or by knocking out the gene is a common practice. To complete this process, various techniques such as CRISPR/Cas9, RNA interference have been performed [101,102].

6.2.1. CRISPER/CAS

CRISPR/Cas9 is one of the precise and efficient tools used in gene modification, especially for the knockout or knockdown process of a particular gene [103]. The first-generation CRISPER gene technology has been utilized for the site-specific cleavage of double-standard DNA [104]. The second generation leads to precise conversion of or replacement of single base into another without cleaving double-standard DNA [105].

In the recent past, in the changing climatic conditions eruptions of novel pathogens or virulent strains and their sustainable management is immediate need for optimum agricultural productions [106–108]. CRISPR/Cas9 is increasingly recognized as a promising tool for managing plant diseases. Additionally, it has been applied to genetically modify microorganisms to boost their beneficial effects on crops [109,110]. Previously, authors utilized CRISPER/Cas9 technology to control phytopathogens. For example, knockdown of the OsERF922 gene, which is known as an "ethylene responsive factor", reduced the chance of infection of *Magnaporthe oryzae* compared to the wild type [111]. Similarly is the knockdown of ERF922 genes, which are upregulated during the *M. oryzae* infection [111]. The editing in the ERF genes help with resistance in pathogen infections [112]. One study reported a significance of CRISPER/Cas9 technology in generating downy mildew–resistant grapes by editing the MLO-7 gene, which has been upregulated during infection of *Erysiphe necator* [113]. Paula de Toledo Thomazella et al. [114] reported the significance of CRISPER/Cas9 technology in editing the SlDmr6-1 gene in tomato, which is responsible for providing resistance against different phytopathogens. Further, Wang et al. [115] reported on the knockout of the VvWRKY52 gene through CRISPER/Cas9 technology, which provided resistance against *Botrytis cinerea* in grapes. Hu et al. [116] reported on the knockout of the CsLOB gene, responsible for bacterial canker diseases in grapefruit. Similarly, knockdown of CsLOB1 in Wanjincheng Orange provided resistance against bacterial canker diseases. Prihatna et al. [117] utilized CRISPER/Cas9 technology to inhibit the Solyc08g075770 (CYCLOPS) gene, which after inhibition enhanced the resistance against the *fusarium wilt* in tomato. Similarly, Paula de Toledo Thomazella et al. [114] reported broad spectrum disease resistance in tomato via CRISPER-cas9–mediated mutagenesis of DMR6 ortholog.

6.2.2. RNA Interference (RNAi)

RNAi is a gene-silencing approach followed to inhibit or silence the gene regulation by inhibiting the functional aspect of target mRNA. During this process, ds RNA is processed into small interfering RNA (siRNA), which along with some specific proteins bind with the target mRNA and cleaves that specific viral or pathogenic mRNA to control the pathogenicity [118–120]. Although for the phytopathogen control, different gene-silencing approaches such as host-induced [121] or spray-induced [122] has been frequently followed.

In the recent past, the scientific community successfully utilized this RNAi technology to inhibit the pathogenicity or protect the crop from phytopathogens including fungi, virus, nematodes, etc. Previous authors have reported on the role of RNAi technology in controlling or inhibiting pathogenicity. Baum et al. [123] reported RNAi in the insect by feeding or injecting ds RNA. However, some of the authors targeted insect genes

by expressing dsRNA in the transgenic plants [124,125]. Koch et al. [122] used RNAi technology to control the infection of *Fusarium graminearum* in barley by spraying CYP3-dsRNA. Mao et al. [126] enhanced the resistance in cotton plant against the cotton bollworm by expressing CYP6AE14 dsRNA. However, utilization of RNAi technology has certain limitations as a gene-silencing approach; for example, the RNAi tool may not be able to target nucleic noncoding RNA or the non-coding RNA present in the nucleus [127]. In addition to this, the RNAi tool may not be able to differentiate the target mRNA or not target the mRNA having very few similarities with the target RNA [128].

7. Future Prospects and Recent Developments

Microbiome engineering is a constantly developing field and is receiving attention from multiple disciplines thereby incorporating novel approaches. However, to engineer the microbiome, multiple hurdles have to be encountered based upon the feasibility and scale-up caliber of the approach. The following section has tried to uncover the newer approaches and their limitations in microbiome engineering.

7.1. Role of Exudate Metabolites

Plants and microbes have co-evolved. The reciprocal benefit to plant and microbes is mediated by means of secondary metabolites. Plants secrete a number of volatile compounds, phytohormones, signaling molecules, extracellular enzymes, and surface receptors in their root exudate, possessing the potential to interfere with their microbial interaction in a positive direction [129]. The potential of root exudates can be harnessed to engineer a rhizospheric microbiome and ultimately increase plant productivity. The selected addition of root exudate can specify the microbiome growth and nutrition acquisition direction [130]. In their study, Jin et al. [131] deciphered that maize seedling exudates promoted growth and reduced oxidative stress in PGPB *Pseudomonas fluorescens.* However, this approach is associated with certain limitations including difficulty in scale-up and identification of specific metabolites in the root exudate.

7.2. Use of Soil Probiotics

Various research is continuously being conducted to understand the impact of probiotics on the human gut microbiome [132]. Similar studies on the impact of external microbial inoculation on the soil and plant microbiome is warranted. PGPM inoculation is a traditional method to increase plant productivity and control plant pathogens [133]; however, their substantial role in microbiome modulation has not been studied much. Recently, Liu et al. [134] demonstrated that inoculation of *Rhodobacter sphaeroides* and *Bacillus amyloliquefaciens* in oilseed rape rhizosphere increased the growth of bacteria belonging to *Pseudomonadaceae* and *Flavobacteriaceae*, leading to better soil nutrient cycling. Similarly, the inoculation of *Burkholderia vietnamiensis* modified soil microbiota and controlled root-knot nematode infestation in watermelon [135]. Nevertheless, this approach is also associated with traditional challenges of competition from the native microbiota. Alternatively, combined approaches of root exudate and PGPR inoculation can be used for a better adaptation to a localized niche.

Increasing agriculture productivity under the pressure of climate change has asked to add another dimension in microbiome modulation. A prompt understanding between synthetic biology, microbiome engineering, and ecology will not only help in climate conservation, but will also increase plant productivity. Harnessing cultured and non-cultured resilient microbes combined with climate smart agriculture can help in combating emerging climatic biotic and abiotic stresses [135]. Epstein et al. [136] explained the role of

microbiome modulation in harboring climate resilience in corals, providing some inputs for microbiome engineering in the soil and plant rhizosphere.

7.3. Optimization of Inoculant Size and Growth Conditions Under In Situ Conditions

Microbiome manipulation is directly correlated with the inoculum size and factors encountered in the specialized niche [9]. Whether artificial microbial consortia, traditional soil amendments, microbiome breeding [137] and transplantation of the microbiome, or modification of root architecture and exudates, every approach needs to be optimized for successful application [138–140]. Seed bio-priming [141], seed coating, encapsulation, nanoformulations [142], and in situ microbiome engineering are some of the latest approaches used for sustained delivery of microbial formulations [143,144]. Nevertheless, further research is required for homogenous optimization of microbial inoculation.

7.4. Role of NGS, Metagenomics of Uncultured Microbiome, and Use of Genetic Tools

Recent advancements in molecular biology can offer important strategies in microbiome modulation. The firm knowledge of microbiome molecular ecology and interactions is necessary for successful manipulation. Next-generation sequencing (NGS) for studying microbial transcriptome, proteome, and metabolome has revealed dynamic changes during plant–microbe interactions [145–147]. High-throughput sequencing of PGPB *Pseudomonas putida* RA-responsive microRNAs and *Bacillus amyloliquefaciens* revealed their role in the growth and development of *Arabidopsis* and the growth of rice under stress [147,148]. Some of the microbes are difficult to culture under laboratory conditions. Metagenomics approaches including high-throughput sequencing, marker gene tags (iTAG), amplicon sequencing, and in situ metagenomics engineering can help in understanding the complexity level and intra-community level interactions [94,149]. Further, genetic editing tools such as miRNA, siRNA-based tools, RNAi, CRISPR/CAS9, and multiomics approaches can be combined with microbiome engineering for increasing plant productivity.

8. Conclusions

In recent decades, to ensure food security for the rising global population, enhancement in agricultural productivity and improved plant performance under stress conditions are some of the main goals. The better understanding of plant–microbe interactions can be a vital option to use the beneficial microbial species as plant or soil inoculants or their use to modulate rhizosphere functioning. Although the isolation, characterization, and selection of beneficial microbial species is complex process, the latest omics and technology make this hurdle easier and help researchers in the identification of microbial communities and their hidden potential. But survivability of the microbial strains in the field trail is one of the challenging tasks. The identification of key genes, microbial engineering, and rhizosphere engineering could be emerging as one of the sustainable solutions to enhance plant performance.

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Perspective

Antibiotic Stewardship in Silkworms: Navigating the Pros and Cons

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Abstract: To safeguard against microbial diseases and maintain optimal silk yields, the sericulture industry relies on antibiotics to promote the health, well-being, and vitality of silkworms (*Bombyx mori*). Antibiotics are commonly incorporated into synthetic diets for rearing silkworms or included as key components in bed disinfectant formulations. The silkworm-rearing industry's reliance on antibiotics has led to concerns about the development of antibiotic-resistant bacteria. Previous research has uncovered a dual role for antibiotics: not only do they prevent disease, but they also promote silkworm growth by reshaping the gut microbiome and enhancing nutrient absorption, highlighting the need for judicious use. Therefore, there is a critical need for prudent management and further exploration of alternative growth-promoting strategies to minimize resistance risks. This study investigates the relationship between antibiotic administration and silkworm growth, shedding light on the mechanisms underlying antibiotic-induced effects and assessing the risk of antimicrobial resistance (AMR) emergence and dissemination.

Keywords: synthetic diets; silkworm rearing; bed disinfectant; mulberry leaves; gut microbiota

1. Introduction

Silkworms (*Bombyx mori* L.) are well-known monophagous insects that exclusively feed on mulberry leaves and are exploited globally to strengthen the sericulture industry. Mulberry leaves are the sole source of nutrition for silkworms' growth and development [1]. Almost 70% of silk is produced with the help of proteins found in mulberry leaves [2]. However, the quality and quantity of mulberry leaves consumed positively correlate with larval growth and cocoon yield, which is only up to a specific limit. Mulberry leaves with low nutritional value cannot meet the requirements for high-quality silk production. The quality of the silk depends on several factors, such as cocoon reelability, percentage of raw silk, non-breakable filament length, tenacity, elongation, cleanness, etc. Domestication has compromised silkworm resilience, leading to inbreeding depression, heightened disease susceptibility, and vulnerability to agro-climatic fluctuations, posing significant challenges to the sericulture sector [3]. The alterations in metabolic profiles and enzymatic activities due to microbial infections negatively affect silk yield and quality [4]. Infection during the rearing stages significantly reduces silk crop yield, causing substantial financial loss to the sericulture industry. The ingestion of contaminated mulberry leaves facilitates horizontal transmission of pathogens to silkworm larvae, posing a significant risk to their health. Notable crop losses have been attributed to various pathogens, such as *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Serratia* sp., *Micrococcus* sp., *Bacillus* sp., etc. [5]. Therefore, it is vital to improve rearing conditions, silk seed and disease

management practices, mulberry leaf quality, etc., to achieve successful rearing. One effective method to enrich silkworm food and improve silk quality is to fortify mulberry leaves. Mulberry leaves have been fortified with nutrients like proteins, amino acids, vitamins, minerals, and carbohydrates, along with various antibiotics [6–9]. Antibiotics play a crucial role in sericulture, with functions encompassing disease prevention, growth promotion through feed additives, and bed disinfection during the rearing process [10]. Oral supplementation of antibiotics with mulberry leaves can ameliorate silkworm growth, biochemical parameters, and silk content [11]. Sericulture has historically utilized broad-spectrum antibiotics like penicillin, tetracycline, streptomycin, chloramphenicol, and others to control bacterial infections [12].

Despite their benefits in growth promotion and disease management, antibiotics' adverse effects on silkworms necessitate rigorous monitoring and regulation in sericulture. Earlier research has shown that antibiotics can interrupt the balance of the silkworm gut microbiome [13], leading to impaired digestion and compromised nutrient absorption, thereby exhibiting adverse effects on the growth and development potential of *B. mori*. Antibiotics may induce oxidative damage to intestinal cells and foster the proliferation of antibiotic-resistant bacteria, thereby limiting therapeutic options for future infections (Figure 1). High antibiotic dosages can trigger stress responses, offsetting growth enhancement and immunoprotection. Therefore, investigating antibiotic–gut microbiome interactions and resistance dynamics in *B. mori* is vital for developing targeted, sustainable sericulture interventions. Our study explores insights into how antibiotic-induced growth promotion in *Bombyx mori* L. relates to the development of resistance, aiming to promote more sustainable and effective practices in sericulture.

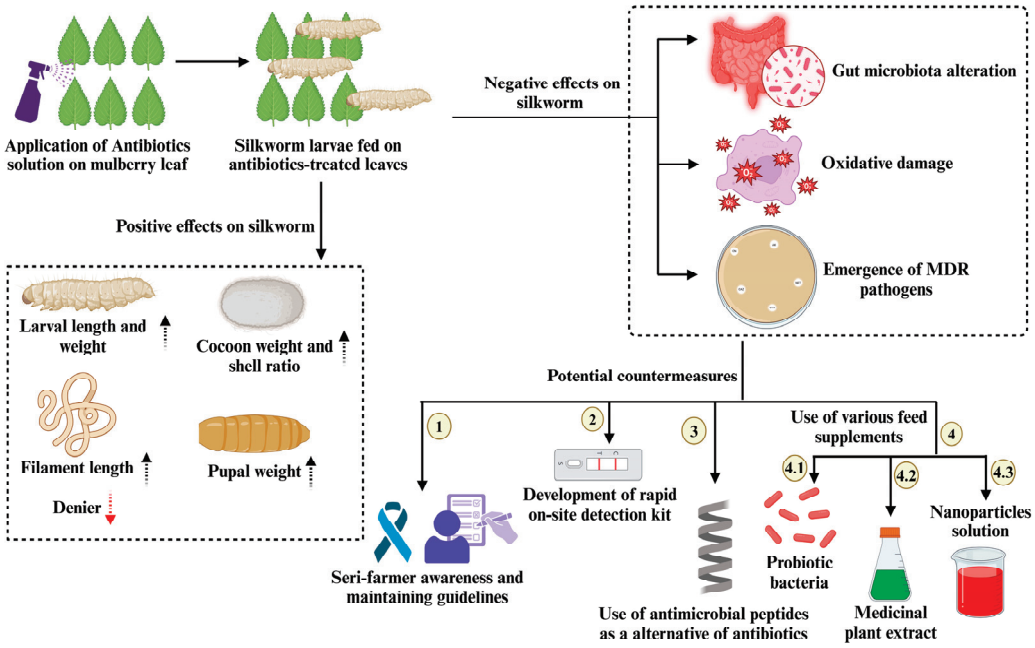

Figure 1. Illustrative model of antibiotic impacts on silkworm physiology and potential countermeasures to optimize sericulture sustainability [black dotted arrow indicates 'increase' and red dotted arrow indicates 'decrease'].

2. Positive Impact of Antibiotics on Silkworm

2.1. Growth Promotion

The administration of antibiotics has been shown to enhance the growth parameters of silkworms by hindering the progress of pathogenic microorganisms and reducing the

mucous membrane's thickness, leading to more nutrient absorption [9]. In addition, antibiotic supplementation in silkworms can also enhance oxygen absorption in the silkworm gut and positively regulate the intestinal flora and physiology. This modulates the microbiota composition, selectively promoting beneficial bacteria while inhibiting pathogenic microorganisms. resulting in growth promotion and silk production [14]. Antibiotic supplementation at different concentrations significantly improved the rearing and cocoon production parameters [15]. For instance, mulberry leaves supplemented with amoxicillin, oxytetracycline, and doxycycline elicited significant increases in the weight of larvae and pupae, including increased body weight and physiological performance metrics [16]. Javaid et al. (2024) also suggest that penicillin-treated mulberry leaves can improve larval weight, larval length, and cocoon production [17]. Earlier studies revealed that chloramphenicol, tetracycline, and amikacin can enhance the cocoon, pupal, and shell weights [18,19]. Cocoon quality metrics, including filament length and shell ratio percentage, exhibited significant improvements following antibiotic treatment. Prior investigations have elucidated the role of antibiotic supplementation in enhancing nitrogen metabolism in silkworm [20]. A concise summary of the effects of antibiotics on silkworms is presented in Table 1.

Table 1. A summary of key findings on antibiotic–silkworm interactions.

Antibiotic	Dosage	Effect on Silkworm	Reference
Ceftiofur sodium Oxytetracycline Enroflaxcin	0.05%, 0.10%, and 0.15%	The weight of the larvae, silk gland, cocoon, and shell increased, along with filament length, cocoon yield, shell ratio, effective rearing rate (ERR), and raw silk percentage, while the denier decreased.	[21]
Oxytetracycline Amoxicillin Doxycycline	1%, 2%, and 5%	The weight of the larvae and pupae increased while the mortality rate decreased.	[16]
Norfloxacin	50 ppm, and 100 ppm	The weight of the silk gland, larval, shell, and cocoon increased.	[11]
Chloramphenicol	25 ppm, and 50 ppm	Enhanced shell content and silk production.	[22]
Amoxicillin	1%, 3%, and 5%	Enhanced cocoon weight, shell content, filament length, and raw silk percentage.	[23]

2.2. Disease Management

Silkworms are highly susceptible to environmental, nutritional, and microbial factors, resulting in various diseases and ultimately leading to mortality and crop losses throughout the year. Pathogenic microbial infections in *B. mori* L. induce a metabolic shift that affects enzymatic activities and reduces silk quality and productivity [17]. Antibiotics show therapeutic and healing effects in *B. mori* larvae. Antibiotics such as penicillin, ampicillin, and streptomycin have effectively reduced silkworm mortality by 23–25% [24]. An earlier study suggested that rifampicin can significantly reduce the mortality of nuclear polyhedrosis virus-infected silkworms [25]. Additionally, it was also observed that the antibiotics, viz., amoxicillin, gentamycin, tetracycline, streptomycin, and kanamycin, supplemented with mulberry leaves resulted in a significant reduction in the occurrence of both grasserie and flacherie diseases [26]. Moreover, Table 2 summarizes various antibiotics and their minimum inhibitory concentration (MIC) values employed to treat pathogens affecting silkworms.

Table 2. Types of antibiotics and their targeted silkworm pathogens.

Antibiotics	MIC (μg/mL)	Targeted Pathogens	Reference
Vancomycin	1	*Staphylococcus aureus*	[27]
Dalbavancin	0.5		
Teicoplanin	1		
Kanamycin	8	*Staphylococcus aureus*	[28]
Arbekacin	8		
Teicoplanin	0.5		
Vancomycin	1		
Tetracycline	0.5		
Minocycline	0.4	*Stenotrophomonas maltophilia*	
	1		
Chloramphenicol	16	*Staphylococcus aureus*	
Flomoxef	0.4		
Linezolid	4		
Sulfamethoxazole-trimethoprim (ST)	256	*Stenotrophomonas maltophilia*	
Imipenem-cilastatin (IPM/CS)	256		
Amphotericin B	3.2	*Candida tropicalis*	
	1.6	*Candida albicans*	
Fluconazole	1.6	*Candida tropicalis*	
	0.4	*Candida albicans*	

3. Negative Impact of Antibiotics in Sericulture

3.1. Oxidative Damage and Gut Microbiota Alterations

Antibiotic supplementation elicits oxidative intestinal toxicity and gut microbiota dysbiosis in silkworms. The antibiotic-induced oxidative damage in silkworms occurs through the down-regulation of crucial antioxidant enzymes (like superoxide dismutase, catalase, glutathione S- transferase, and thioredoxin reductase). Antibiotic exposure elicited transcriptional alterations in antimicrobial peptide genes (attacin, lysozyme, and cecropins), compromising intestinal immune function and promoting intestinal microbiota imbalance in silkworms [13]. This led to enhanced representation of specific bacterial genera *Acinetobacter*, *Bacteroides*, *Barnesiella*, *Bradyrhizobium*, *Burkholderia*, *Escherichia*, *Phenylobacterium*, *Plesiomonas*, *Shigella*, *Sphingobium*, including some unclassified bacteria, with a notable reduction in *Enterococcus*. Such antibiotics disrupted the *B. mori* microbiota, impacting metabolic pathways, enzyme activities, and functional profiles [29].

3.2. Emergence of Antibiotic-Resistant Microorganisms

During antibiotic therapy, the *B. mori* microflora is exposed to the antibiotic's selection pressure, leading to the development of antibiotic-resistant strains. These resistant silkworm bacterial strains carry genetic reservoirs with the potential to disseminate antibiotic resistance genes (ARGs) to other strains [30] via horizontal gene transfer (HGT) and vertical gene transfer (VGT), exacerbating antibiotic resistance [31]. Plasmid-mediated conjugation facilitates the process of HGT of ARGs from the infected mulberry leaves to the silkworms. The silkworm gut, with its diverse microbiota, serves as a bioreactor for developing antibiotic resistance. Later, the ARGs are excreted in the silkworm feces and eventually end up in the soil. The colonization of ARG-carrying bacterial pathogens in plant roots and their upward transmission to aerial organs is facilitated by the presence of lateral roots, whose epidermal remodeling creates susceptible entry points (Figure 2) [31,32]. The overlap between silkworm and human pathogens necessitates enhanced biosecurity protocols and surveillance to break the disease outbreak loop from silkworms to humans [33,34].

In a previous study conducted by Javaid and his team, six pathogenic bacterial strains were successfully isolated from infected silkworm larvae. The researchers found that most of these strains (83.3%) demonstrated a high frequency of antibiotic resistance against a panel of antibiotics, viz., penicillin, tetracycline, amoxicillin, ampicillin, and erythromycin. In contrast, only 16.6% of the isolated strains showed susceptibility to these antibiotics. Notably, the screened bacterial strains showed a close phylogenetic relationship with established silkworm pathogens such as *Bacillus halotolerans*, *Pseudomonas aeruginosa*, and *Serratia marcescens* [17]. In our previous study, Mondal and his team successfully isolated multidrug-resistant (MDR) strains of *Mammaliicoccus* sp. and *Pseudomonas* sp. from the hemolymph of infected silkworm larvae [35]. Building on our research, we conducted a separate study where we successfully isolated and identified a multidrug-resistant bacterial strain, *Enterobacter* sp., from infected silkworm larvae, highlighting the diversity of MDR pathogens affecting sericulture [36] and underscoring the necessity for innovative management strategies and adjunctive therapeutic modalities to combat the emergence of these resistant pathogens and ensure optimal silkworm health.

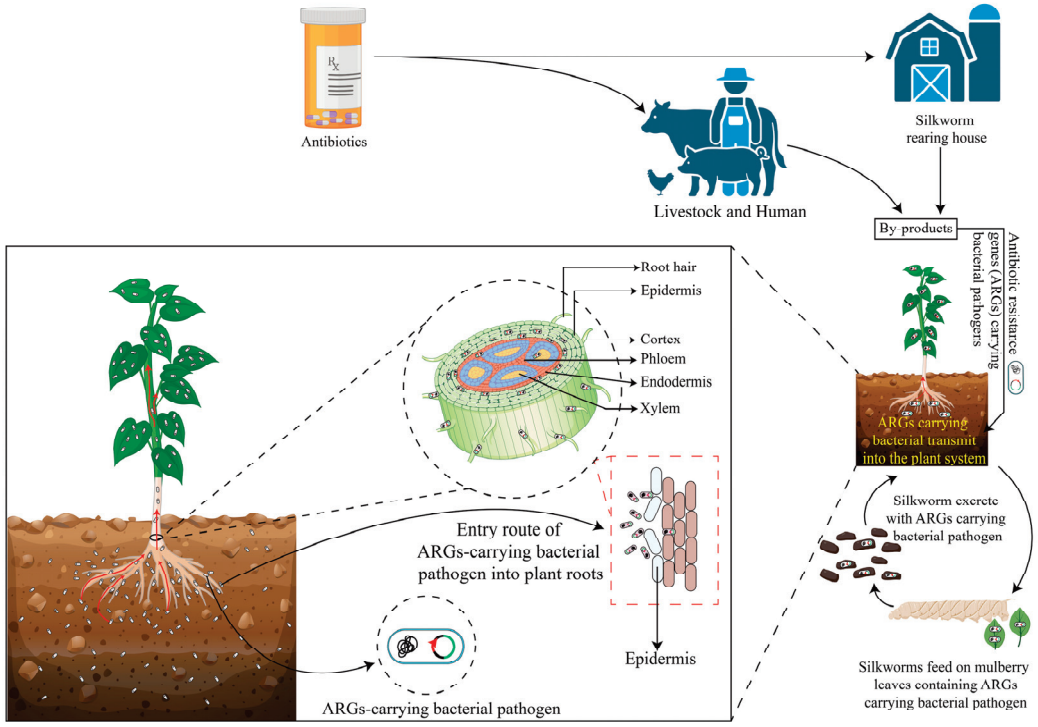

Figure 2. A thumbnail sketch of the rise of ARG-carrying MDR pathogens in silkworms.

4. Mitigation Strategies for Minimizing the Consequences of Antibiotic Overuse

Multiple strategies based on antibiotic overuse can be employed to reduce the negative impacts of antibiotic misuse in sericulture, as discussed below.

4.1. Implementing Antibiotic Stewardship Programs (ASPs)

The alarming rise of antibiotic resistance necessitates a sustained, collaborative response from stakeholders. The intensive uncontrolled use of antimicrobials in sericulture has led to the emergence of MDR pathogens, posing significant risks to *Bombyx mori* L. and human health, as well as environmental and food chain safety [37]. To combat this growing threat, implementing Antibiotic Stewardship Programs (ASPs) is crucial. Effective stewardship practices promote responsible antibiotic use, reduce antibiotic-resistant infections,

and protect public health. By adopting ASPs, stakeholders can mitigate the risks associated with antibiotic resistance. ASPs are crucial for encouraging the controlled use of antibiotics. These initiatives aid the education and awareness of farmers by providing training on judicious antibiotic application, clarifying when such treatments are necessary, and how to avoid unnecessary antibiotic usage. By focusing on judicious antibiotic administration, farmers can enhance the overall welfare of their livestock, reducing mortality rates and boosting productivity. Furthermore, these programs emphasize the optimization of dosages and duration by establishing guidelines that minimize the risks of antibiotic resistance and the adverse effects linked with it [38–43].

4.2. Enhancing Diagnostic and Waste Management Practices

Globally, AMR is one of the most challenging threats to public health; thus, there is an unmet need for rapid diagnostics to reduce the misuse of antibiotics. Identifying the disease-causing specific pathogens and their AMR patterns is foremost important to minimize unnecessary antibiotic use and control the spread of AMR. Innovations in biosensor techniques have revolutionized the rapid diagnostics practices in sericulture [44]. This early detection enables sericulture farmers to implement control measures before diseases spread widely, safeguarding their crops and investments. By accurately identifying pathogens, farmers can tailor their management practices to address specific silkworm health issues, leading to improved animal welfare and productivity. This targeted approach minimizes the reliance on broad-spectrum antibiotics, promoting optimal silkworm health and resulting in healthier silkworm populations, higher quality silk, and improved yield rates. Adopting effective diagnostic practices enables sericulture farmers to monitor the health of silkworms throughout their life cycle, leading to early disease detection and targeted interventions [45]. This not only benefits the individual farmers but also contributes to the reputation and competitiveness of the silk industry as a whole. By prioritizing these practices, sericulture farmers can protect their livelihoods while contributing to a more resilient and sustainable silk industry. To address disease detection issues, researchers have developed rapid biosensor models based on lateral flow assay (LFA) strips and Loop-mediated isothermal amplification combined with lateral flow dip-sticks (LAMP-LFD) methods to detect MDR bacteria, including *Pseudomonas* sp., *Mammaliicoccus* sp. [36], as well as microsporidian parasites like *Nosema bombycis* [46] and viruses such as *Bombyx mori* bidensovirus (BmBDV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) [47,48]. Additionally, improving the disposal management of silkworm by-products is essential to reduce antibiotic residues entering the environment, which can contribute to resistance development in natural ecosystems [13]. Antibiotic residues from silkworm farming can contaminate soil and water, alter microbial flora, harm beneficial soil microbes, and promote the growth of antibiotic-resistant bacteria, posing risks to both environmental and human well-being. Furthermore, silkworm feces can introduce antibiotic-resistant genes (i.e., rpoB, oqxA, and qnrB) into ecosystems, affecting other organisms and humans. The most common antibiotic resistance genes (ARGs) found in feces confer resistance to multiple drugs, β-lactam antibiotics, rifamycins, peptide antibiotics, and quinolone antibiotics. This suggests that the fecal microbiome harbors a diverse array of resistance mechanisms, posing potential risks to human health [31]. While antibiotics are used in sericulture for disease management, improper feces disposal can exacerbate antibiotic resistance. Therefore, effective waste management is crucial to minimize the emergence of MDR bacteria and the associated risks.

4.3. Antimicrobial Peptides as an Alternative to Traditional Antibiotics

The alarming increase in antibiotic resistance necessitates the development and implementation of alternative therapeutic strategies to mitigate the spread of resistant pathogens and address the limitations of traditional antibiotic treatments. Recent studies have highlighted the potential of antimicrobial peptides (AMPs) derived from natural sources as effective substitutes for antibiotics, offering a sustainable solution to mitigate antibiotic overuse and resistance [49]. AMPs are low-molecular-weight oligopeptides that play a pivotal role in the host's innate immune response and have broad-spectrum antimicrobial activity [50]. These peptides possess secondary structures such as α-helices, β-strands, β-hairpins or loops, or an extended conformation. AMPs destroy the target cells by forming pores and disrupting cell membranes. The general mode of action by the AMPs is (i) attraction of AMPs towards the target cell, (ii) attachment of AMPs to the plasma membrane of the target cell, and (iii) inclusion of AMPs into the lipid bilayer of the target cell, resulting in the formation of leaky pores that allow leakage of the cytosolic content. This mechanism is primarily explained through three models: the barrel-stave model, the carpet model, and the toroidal-pore model [51]. Cationic AMPs exhibit potent bactericidal activity through electrostatic attraction and binding to the anionic cell surface of target microorganisms, facilitating membrane disruption and cell lysis Unlike conventional antibiotics, microorganisms cannot develop resistance to AMPs [52]. *Bombyx mori*, the silkworm, has evolved an innate immune response involving the production of antimicrobial peptides (AMPs) to counter microbial infections, offering a unique opportunity for identifying novel AMPs with therapeutic potential [53,54]. Various classes of silkworm-originated AMPs, such as cecropins, lebocins, attacins, enbocins, defensins, gloverins, moricins, and ponericins, have shown broad-spectrum antimicrobial activity against bacteria, fungi, and viruses, indicating their potential therapeutic benefits [51,52]. Moreover, combining AMPs with existing antibiotics can enhance efficacy, allowing for reduced antibiotic dosages and minimizing the risk of antibiotic overdose and associated resistance issues [55–57]. Additionally, AMPs can be integrated into silkworm feed or their rearing environment to boost immune responses, thus lowering the incidence of infections that require antibiotic treatment [58,59]. Overall, incorporating AMPs into disease management strategies can significantly reduce reliance on traditional antibiotics, promoting healthier and more sustainable sericulture practices.

4.4. Introducing Various Feed Supplementation as Silkworm Growth Enhancer

4.4.1. Probiotic Bacteria as Feed Supplementation

Probiotics have grabbed significant attention in animal husbandry due to their beneficial impact on animal growth and health. Feed supplemented with probiotics, particularly lactic acid bacteria (LAB), significantly improves silkworm health. LAB such as *Lactobacillus paraplantarum* stimulate the immune responses in the silkworm by promoting cytokine release, thereby improving survival rates against pathogens, including *Pseudomonas aeruginosa* [60]. Probiotics also enhance digestive health by promoting a balanced gut microbiome, improving feed breakdown and nutrient absorption, and leading to better growth rates. A recent study suggests that the probiotic strain *Pediococcus pentosaceus* promoted silkworm growth performance metrics, such as body weight, feed efficiency, and silk yield, through alterations in the activity of intestinal digestive enzymes and nutrient supplementation (e.g., Vitamin B). Additionally, *P. pentosaceus* supplementation elicits the antioxidant level and produces antimicrobial substances that protect the silkworm from pathogens. Furthermore, the strain alters gut metabolite levels and microbiota, ensuring balanced nutrition and optimal health of the host [61]. Suraporn et al. (2015) observed that *Lactobacillus acidophilus* enhances various growth parameters in silkworms (such as larval weight, survival rate,

cocoon weight, pupation rate, and cocoon shell ratio) and ultimately boosts the silk yield. The presence of *L. acidophilus* in the silkworm mid-gut benefits the host by improving digestion and food assimilation, which in turn leads to increased growth rates and higher silk production [62]. Similarly, Pachiappan et al. (2021) suggest that supplementing the diet of silkworms with 3% probiotic microorganisms such as *Saccharomyces boulardii*, *Lactobacillus rhamnosus*, and *Bifidobacterium longum* can positively alter their enzymatic (amylase and invertase) activity and enhances the cocoon weight, pupal weight, shell weight, and shell ratio [63]. Overall, the use of probiotics in silkworm diets offers multiple benefits, including enhanced immunity, improved pathogen resistance, and greater digestive efficiency. Collectively, these factors synergize to enhance the overall viability and productivity of the silkworm, ensuring optimal growth, development, and silk production [64].

4.4.2. Medicinal Plant Extract as Feed Supplementation

Medicinal plant extracts as feed supplements for silkworms are gaining attention due to various beneficial effects on silkworm growth, health, and silk production. Fortifying mulberry leaves with plant extracts enhances the overall nutritional status, leading to better growth and development of the larvae. Earlier studies showed that dietary supplementation of mulberry leaves with *Ocimum sanctum* extract (2–3%) exhibited positive impacts on silkworm growth, cocoon quality, and silk production [65,66]. Similar results were also found in the case of 3% *Aloe vera* and *Withania somnifera* extract [66]. According to Hassan et al. (2018), fortifying mulberry leaves with *Moringa oleifera* extract has numerous benefits for silkworm cultivation. This supplementation significantly enhances cocoon weight, shell weight, and filament length, ultimately improving silk production. Additionally, *Moringa oleifera* extract boosts enzymatic activity, specifically protein transaminases and protease enzymes, which promote protein metabolism. Notably, the extract also exhibits therapeutic activity against bacterial infections in silkworms, reducing susceptibility to disease [67].

4.4.3. Nanoparticles (NPs) Solution as Feed Supplementation

Rearing silkworms with a nanoparticle-supplemented diet effectively enhances the quality and quantity of silk cocoons, as well as fiber quality. A wide range of nanoparticles, like metal nanoparticles such as silver (Ag), gold (Au), and titanium dioxide (TiO_2) nanoparticles, and carbon-based nanomaterials such as single-walled carbon nanotubes (SWNTs) and graphene, have been studied for their impact on silkworms [68–70]. Earlier studies suggest that silkworm larvae fed with AgNPs beneficially influence larval weight, pupal survivability, cocoon and shell weight, filament length, and quantity of silk production by enhancing the feed efficacy [71–73]. Cai et al. (2015) observed that in vivo uptake of TiO_2 NPs by silkworms led to the direct production of ultraviolet-resistant silk. TiO_2 NPs were incorporated into the silk gland to induce a conformational transition of silk fibroin from random coil/α-helix to β-sheet structure [68]. Additionally, silkworms fed with TiO_2 NPs showed a 14.88-fold resistant property against BmNPV relative to control larvae fed with sterile water-treated mulberry leaves [74]. Furthermore, it was also observed that silkworms fed with SWNTs and graphene demonstrated improved elongation and toughness via increasing elongation at break and toughness modules [69]. In our previous study, Mondal and his team observed that AuNPs enhance both the rearing and reeling performances of silkworms without any adverse side effects compared to the control group [35]. However, further research is necessary to elucidate the size- and concentration-dependent toxicity risks associated with nanoparticle exposure in silkworms. Future research should focus on optimizing nanoparticle formulations for safe and effective use in sericulture practices.

5. Conclusions

The benefits of antibiotics in silkworm cultivation are countered by the risk of resistance, which jeopardizes silkworm health, disrupts ecological harmony, and complicates disease management, emphasizing the need for judicious antibiotic use. To address these issues, a multi-pronged approach of maintaining hygiene, improving rearing practices using disease-resistant silkworm breeds, and judicious antibiotic usage is needed to prepare for and manage AMR pathogens in sericulture-based industries. Collaboration between farmers, researchers, and public health authorities is the key to mitigating the risks associated with such pathogens. The stakeholders may also develop comprehensive strategies that could protect public health from emerging infectious diseases, especially when driven by changes in host species. Nowadays, modern research is focused on the newly engineered and natural anti-infective agents that can aid in combating AMR pathogens more effectively. Moreover, the discovery and deployment of rapid, portable, and on-site disease detection platforms like lateral flow assay (LFA)-based biosensors should be the major thrust area in this field to revolutionize sericulture-based industries.

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Review

Application of Biofertilizers for Enhancing Beneficial Microbiomes in Push–Pull Cropping Systems: A Review

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Abstract: The need for sustainable farming practices has brought attention to biofertilizers to improve soil quality and boost crop yield while minimizing environmental impacts. This study explores the potential integration of biofertilizers within push–pull cropping systems, an agroecological approach that utilizes companion cropping to repel and attract pests. This review focuses on how biofertilizers could optimize plant–microbe interactions, promoting nutrient uptake, pest control, and soil health. Key biofertilizers, including nitrogen-fixing, phosphate-solubilizing, and potassium-solubilizing bacteria, improve nutrient availability, which leads to higher crop yields and resilience. They also enhance soil water retention and drought tolerance, which are crucial under changing climate conditions. Biofertilizers support beneficial microbial communities, reducing reliance on synthetic fertilizers and pesticides while fostering disease suppression and stress tolerance in crops. Their effectiveness can be significantly increased when biofertilizers are integrated with push–pull technology (PPT). However, challenges remain, such as inconsistent biofertilizer performance and the complexity of microbial interactions. Overcoming these challenges necessitates a multidisciplinary approach to refining production and application techniques. This study emphasizes the need to investigate biofertilizer-mediated plant–microbiome dynamics further to unlock their full potential. It concludes that future research should focus on the synergies between biofertilizers and agroecological systems to enhance food security and environmental sustainability. This work advances our understanding of optimizing biofertilizers in sustainable farming practices, particularly within the PPT framework.

Keywords: biofertilizers; push–pull technology; sustainable agriculture; nutrient uptake; soil health; plant–microbe interactions

1. Introduction

The rising need for producing food and the negative effects on the environment caused by farming methods have sparked a focus on sustainable agriculture practices. Recently, the application of biofertilizers in crop production has gained prominence as a sustainable approach to enhancing soil health and crop productivity [1,2]. Among various agroecological practices, push–pull cropping systems have emerged as a highly effective method for managing pests, improving soil fertility, and boosting crop yields [3–6]. Push–pull technology (PPT) involves the strategic use of trap and repellent crops to manage pest populations while enhancing soil health through the incorporation of beneficial microorganisms [7]. Despite its proven benefits, the effectiveness of push–pull systems can be significantly enhanced by the application of biofertilizers, which support and amplify beneficial microbiomes in the plant–soil continuum [8,9]. However, to fully realize the potential of biofertilizers in PPT, it is essential to understand the mechanisms underlying their interactions with crops and associated microbial communities [10].

Push–pull technologies have demonstrated their ability to improve crop productivity and manage pest infestations in various farming contexts [7]. These systems leverage

plant–microbe interactions to create more resilient agricultural environments. Biofertilizers, including phosphate-solubilizing bacteria (PSB) [11], potassium-solubilizing bacteria (KSB) [12], and nitrogen-fixing bacteria (NFB) [13], play a crucial role in optimizing these interactions by enhancing nutrient availability and supporting microbial communities. Research has shown that biofertilizers significantly improve soil properties and plant health by fostering beneficial microbiomes that contribute to nutrient cycling, disease suppression, and stress tolerance [14–18]. The ability of biofertilizers to stimulate plant growth and defense responses further amplifies the benefits of PPT, leading to improved crop yields and reduced reliance on synthetic inputs [10]. However, successfully integrating biofertilizers into PPT hinges on understanding the intricate relationships between the crop, the associated microbial community, and surrounding environmental factors.

Recognizing these complexities, researchers have made extensive efforts to elucidate the mechanisms by which biofertilizers enhance the performance of PPT. Key mechanisms include the direct promotion of plant growth through phytohormone production, enhanced nutrient availability via biological nitrogen fixation and phosphate solubilization, and the suppression of plant pathogens through antimicrobial compound production and the induction of systemic resistance [10,18]. For instance, PSB increase phosphorus availability, a critical nutrient for plant growth [11,19–21], while KSB improve potassium availability, vital for crop resilience [12,22]. Similarly, NFB contribute to soil nitrogen content, promoting plant growth and yield [23]. Integrating these biofertilizers within PPT can enhance their effectiveness and support sustainable agricultural practices by reducing reliance on chemical fertilizers and improving soil health. Therefore, the strategic use of biofertilizers in PPT holds great promise for advancing sustainable and resilient agricultural systems [10,24–26]. However, a deeper understanding of biofertilizer-mediated changes in plant–microbiome interactions is needed to maximize their potential within PPT systems [10,18,25,27].

Furthermore, biofertilizers can improve soil water retention and plant drought tolerance—critical factors in changing climate conditions [8,28]. Recent studies have shown that applying biochar and microbial inoculants can enhance water retention and crop productivity, which can benefit push–pull systems [29,30]. Despite the growing body of evidence supporting biofertilizers in PPT, further research is needed to fully understand how these microorganisms interact with plant and soil systems [31]. The potential for biofertilizer adoption in PPT is hindered by several challenges, including variable and unpredictable performance, the complex interactions between biofertilizers and the soil–plant–microbe continuum, and the lack of robust and reliable production and application methods [10,26]. Overcoming these challenges requires a multidisciplinary approach, bringing together microbiologists, plant scientists, and agronomists to develop innovative solutions. This study aims to explore the application of biofertilizers in enhancing beneficial microbiomes within PPT, focusing on their impact on pest management, soil health, water management, and crop productivity. The research will contribute to optimizing PPT and advancing sustainable agricultural practices by bridging gaps in current knowledge.

2. Push–Pull Technology

Push–pull technology (PPT) is an agroecological strategy that involves the intercropping of companion crops to manage pests without heavy reliance on chemical pesticides [3,5,6,32]. This approach combines "push" crops that repel pests and "pull" crops that attract pests away from the main crop. The most popular PPT practiced in East Africa involves maize or sorghum intercropped with leguminous plants of the genus *Desmodium* (Fabaceae), surrounded by *Napier grass* (*Pennisetum purpureum*) or *Brachiaria* species (both Poaceae) [33]. The *Desmodium* intercrop protects cereal crops by repelling stem borer pests, including the emerging *Spodoptera frugiperda* (fall armyworm), through a "push" effect while attracting parasitoids such as *Cotesia ruficrus*, *Cotesia sesamiae*, and *Trichogramma* spp. These parasitoids further reduce pest populations by parasitizing the pests, laying their eggs on or inside them, with the larvae consuming the host from within [34,35]. Additionally, microbial parasites are crucial in pest management within push–pull systems. For

example, *Bacillus thuringiensis* (Bt) produces toxins that are lethal to specific insect larvae [3], while entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae,* infect and kill insects by penetrating their cuticles and growing inside them [36]. Furthermore, *Pythium* spp., a group of oomycetes, can parasitize insect larvae by infecting their tissues. The peripheral grasses act as a "pull" factor, attracting pests away from the crops for oviposition but preventing full larval development, thereby functioning as ecological traps.

In addition to pest control, PPT provides other agroecosystem benefits, such as reducing the need for synthetic chemical pesticides and fertilizers—an essential step toward sustainable agriculture. The allelopathic effects of *Desmodium* root exudates help control parasitic weeds, such as *Striga hermonthica* [7]. *Desmodium* is a leguminous species that also improves soil health by fixing nitrogen, facilitating carbon sequestration, solubilizing phosphorus, and depositing organic matter [37]. Plant-associated microbiomes further enhance productivity and overall plant health by regulating nutrient availability and uptake, improving tolerance to abiotic stress and increasing disease resistance. The root-mediated mechanisms that promote microbial species diversity in the soil within PPT systems can be further amplified by applying biofertilizers.

3. Beneficial Microbes in the Plant–Soil Continuum

Plant–soil microbiomes are crucial for plant health, stress tolerance, nutrient acquisition, and interactions with agricultural practices (Table 1). Research by [38] has demonstrated that plant-associated microbes enhance plant growth, disease resistance, and overall health by interacting with roots and influencing nutrient uptake. However, the specific mechanisms through which these interactions occur, particularly under varying environmental conditions, remain insufficiently understood. The role of the rhizosphere microbiome in suppressing soil-borne pathogens and enhancing disease resistance through various mechanisms has been well established [39–43]. S. Compant et al. [40] emphasize that the rhizosphere microbiome plays a critical role in plant health by influencing root growth, nutrient uptake, and disease resistance. Another study highlights the complexity of interactions within the rhizosphere, where beneficial microorganisms suppress plant pathogens through various mechanisms, including producing secondary metabolites, competition for nutrients and space, and modulation of the plant immune system [42]. P.A.H.M Bakker et al. [43] show that specific strains of fluorescent *Pseudomonas* can induce systemic resistance in plants, providing enhanced defense against a wide range of pathogens. Their study explains that the bacteria trigger plant immune responses without directly killing pathogens, leading to more robust and sustained disease resistance. S. Compant et al. [40] highlight that those beneficial microbes in the rhizosphere, such as mycorrhizal fungi and nitrogen-fixing bacteria, can help plants tolerate abiotic stresses, like drought and heat, which may also indirectly improve their resistance to pathogens. Nevertheless, more detailed studies are needed to understand how plant species and soil types influence these microbial communities.

Endophytic microorganisms have improved plant tolerance to environmental stresses such as drought, salinity, and metal toxicity [44–46]. For example, *Piriformospora indica* has been demonstrated to enhance drought tolerance in crops, like wheat and barley, by promoting root growth, increasing water and nutrient uptake, and boosting the expression of stress-responsive genes [45]. *Bacillus subtilis* has been found to improve drought tolerance in maize by stimulating the production of plant growth hormones, such as auxins, which increase root biomass and water retention [46]. Similarly, *Pseudomonas fluorescens* and *Azospirillum brasilense* have been reported to enhance salinity tolerance in rice and wheat by improving ion homeostasis, reducing oxidative stress, and facilitating nutrient uptake [44]. *Enterobacter cloacae* has been found to increase plant salt tolerance by producing phytohormones and enhancing antioxidant enzyme activities, which help plants cope with the osmotic stress induced by high salt levels [47]. *Pseudomonas putida* has also been reported to reduce heavy metal toxicity (such as cadmium and lead) in plants by sequestering metals in root tissues and promoting the production of stress-related proteins [48]. Despite these

findings, the precise pathways through which endophytes alleviate these stresses, as well as their potential applications in sustainable agriculture, require further exploration.

Symbiotic relationships between plants and microbes, such as mycorrhizae and NFB, significantly enhance nutrient uptake [49]. Arbuscular mycorrhizal fungi (AMF) have been shown to improve the uptake of phosphorus (P), which is typically immobile in soil. *Glomus intraradices* extend the root system by forming hyphal networks that explore the soil more efficiently than plant roots alone, thereby enhancing P uptake in wheat by facilitating nutrient transport [50]. *Pisolithus tinctorius*, an ectomycorrhizal fungus commonly associated with trees, such as pine, oak, and birch, enhances nitrogen (N) and P uptake, promoting tree growth in nutrient-poor soils [51]. *Rhizobium leguminosarum* forms nodules on pea roots, providing the plant with fixed N in exchange for carbon compounds from the plant [52]. *Azospirillum* is associated with the roots of grasses (maize, wheat, rice, etc.), enhancing N availability and stimulating root growth by producing plant growth hormones such as auxins, gibberellins, and cytokinins [53]. *Herbaspirillum seropedicae* is an endophytic nitrogen fixer associated with sugarcane productivity in low-N soils [54]. However, the genetic and environmental factors that optimize these symbioses for different crops are not yet fully understood.

Agricultural practices such as crop rotation, tillage, and fertilization can significantly alter plant-associated microbiomes [55–57]. Crop rotation, the practice of alternating different crops (e.g., soybean and maize) in the same field, promotes a more diverse soil microbiome. During the legume phase, the populations of *Rhizobium* and *Bradyrhizobium* increase, enhancing N availability and microbial diversity [55]. Additionally, crop rotation can reduce soil pathogen populations, as non-host crops interrupt the life cycles of pathogens specific to certain crops. For instance, rotating cereal crops with brassicas (e.g., mustard) have been shown to reduce populations of soil-borne pathogens, like *Rhizoctonia* and *Fusarium* spp., thereby allowing beneficial microbes to thrive [58]. Conversely, conventional tillage can disrupt microbial communities and reduce the abundance of fungi, such as AMF. In contrast, no-tillage practices promote the growth of beneficial microbes, like *Pseudomonas* and *Bacillus* spp., which support crop growth and enhance disease resistance [59]. The impact of fertilization on the soil microbiome depends on the fertilizer used. Synthetic nitrogen fertilizers alter microbial community composition by favoring fast-growing bacteria that thrive in nitrogen-rich environments while reducing the abundance of nitrogen-fixing bacteria and mycorrhizal fungi [60]. Organic fertilizers, by contrast, enhance soil microbial diversity by providing a slow-release nutrient source and increasing organic matter content. Studies have shown that applying compost to fields increases populations of beneficial microbes, such as *Trichoderma* spp., which can suppress soil-borne pathogens, and promote the growth of phosphate-solubilizing bacteria that improve phosphorus availability to plants [61]. However, there remains a gap in understanding how specific agricultural practices can be optimized to maintain or enhance beneficial microbial communities.

Table 1. Studies on beneficial microbes in the plant–soil continuum.

Microbes Studied	Key Benefits	References
Rhizobia	Nitrogen fixation, improved soil fertility, increased crop yield	[13,18,23,52]
Mycorrhizal fungi	Enhanced nutrient uptake (phosphorus, nitrogen), improved drought resistance, increased crop yield	[50,51,62,63]
Pseudomonas	Disease suppression, enhanced root growth, increased nutrient uptake	[41,43]
Bacillus	Biocontrol of soil-borne pathogens, enhanced plant growth, improved stress tolerance	[17,53,64]
Trichoderma	Biocontrol of plant pathogens, improved seedling vigor, increased crop yield	[16,65,66]
Azospirillum	Nitrogen fixation, improved root architecture, increased nutrient uptake and growth	[13,43,53,66]
Endophytic fungi	Enhanced growth, increased resistance to biotic and abiotic stress	[40,45,67,68]

Table 1. *Cont.*

Microbes Studied	Key Benefits	References
Phosphate-solubilizing bacteria	Improved phosphorus availability, enhanced root development, increased yield	[11,12,20,24,29]
Actinomycetes	Biocontrol of pathogens, enhanced nutrient cycling, improved plant growth	[39,40,66]
Plant growth-promoting rhizobacteria (PGPR)	Enhanced disease resistance, improved nutrient uptake, increased plant growth	[10,39]
Nitrogen-fixing bacteria	Improved nitrogen availability, enhanced plant growth, increased yield	[13,52,69]
Siderophore-producing bacteria	Enhanced iron uptake, improved plant health, increased resistance to pathogens	[10,39,48,66]
Lactic acid bacteria	Improved plant growth, enhanced nutrient uptake, biocontrol of soil pathogens	[47,66,70]
Cyanobacteria	Nitrogen fixation, improved soil health, increased crop productivity	[1,48,71]
Vesicular–arbuscular mycorrhiza (VAM)	Improved nutrient and water uptake, enhanced soil structure, increased plant resilience	[50,51,62,63]

Soil microbiomes are crucial in plant growth, nutrient uptake, overall plant health, and soil structure [31]. Organic farming practices promote greater diversity and activity of soil microbiomes, leading to improved soil health and increased crop productivity compared to conventional farming methods [56,57,61,72]. Over the long term, organic farming significantly influences the diversity and activity of soil microbial communities, enhancing soil health and crop productivity compared to mineral-based fertilization strategies [61]. K. Hartman et al. [56] demonstrated that organic farming boosts the abundance and diversity of soil microbiomes, improving soil health and enhancing crop productivity. Additionally, J. Nelkner et al. [57] reported that organic farming practices lead to a higher diversity and functional activity of beneficial soil microbiomes than conventional farming, resulting in improved soil health and increased crop yields. L. Qiu et al. [72] further showed that organic farming practices can mitigate soil erosion and improve microbial diversity and network complexity, which are essential for maintaining soil health and productivity compared to conventional methods. Specific soil microbiomes can suppress plant diseases by outcompeting pathogens, producing antimicrobial compounds, and inducing plant systemic resistance [14]. Several studies [21,26,68] have highlighted the essential roles of soil microorganisms in nutrient cycling, particularly N, P, and carbon, which are crucial for soil fertility and plant growth. Soil health is closely tied to microbiome diversity, with more diverse microbiomes contributing to improved soil structure, nutrient availability, and disease suppression [41,56]. The activities of soil microbiomes regulate the decomposition of organic matter and soil carbon sequestration [63,68]. Microbial communities can also adapt to degrade pollutants in contaminated environments, enhancing bioremediation processes [40,48,73]. Furthermore, microbiomes contribute to soil stability and resistance to erosion by influencing soil aggregation and structure [72,74]. Microbes' secretion of extracellular polysaccharides promotes soil particle aggregation, thereby enhancing soil structure [56,61]. Additionally, soil microbiomes play a significant role in soil respiration through the decomposition of organic matter and microbial activity, both of which affect soil carbon fluxes [68,72].

4. Applications of Biofertilizers in Push–Pull Technologies

4.1. Biofertilizers

A biofertilizer refers to a microbial inoculant formulation that contains cultures of dormant or live cells from beneficial strains, such as nitrogen-fixing and phosphorus-solubilizing microorganisms [2,70]. From a cellular perspective, biofertilizers are typically applied in three ways: to seeds, plants, or soil. Most biofertilizer applications aim to enable beneficial microorganisms to colonize the rhizosphere or the plant's interior, promoting growth by increasing the supply or availability of primary nutrients to the host plant [75]. Microbiomes beneficial for plant production in biofertilizers are commonly referred to as

plant growth-promoting microbes (PGPMs), plant growth-promoting bacteria (PGPB), or plant growth-promoting rhizobacteria (PGPR) [2]. A recent study detailed the primary mechanisms of biofertilizers, including improved nutrient supply, regulation of plant growth, and enhancement of soil quality and microbiome health [2].

Biofertilizers can have multiple impacts through various roles and mechanisms across the soil–plant continuum, promoting sustainable agriculture (Table 2). In agroecological systems, biofertilizers are integrated into push–pull farming techniques, enhancing beneficial microbial communities while contributing to pest management and soil health [7,76]. Biofertilizers reduce the need for synthetic fertilizers and pesticides, promoting sustainable farming by enhancing soil fertility and plant resilience [17,49,70]. Studies have shown that biofertilizers, such as AMF and vesicular–arbuscular mycorrhizae (VAM), improve soil structure and nutrient availability, thereby enhancing soil quality [62]. Additionally, cyanobacteria have been effective in boosting soil health by improving N fixation and overall soil productivity [71]. PGPR and endophytic fungi have been shown to enhance plant health by increasing nutrient uptake, promoting growth, and improving resistance to biotic and abiotic stresses [67,77]. Specifically, *Pseudomonas* species have proven beneficial in promoting plant growth and controlling soil-borne pathogens, aiding in pest management [41,43]. Collectively, these studies underscore the vital role of biofertilizers in advancing sustainable agricultural practices by improving soil conditions, strengthening plant health, and managing pests.

Table 2. Synthesized insights on the roles and mechanisms of biofertilizers in sustainable agriculture.

Biofertilizer Impact	Key Insights	References
Nutrient uptake enhancement	Nitrogen-fixing bacteria (e.g., *Azospirillum, Rhizobia*) improve nitrogen and phosphorus uptake, reducing dependence on chemical fertilizers.	[20,26,54,77]
Plant growth and yield improvement	PGPRs (*Pseudomonas, Trichoderma*) promote plant growth by producing plant hormones, enhancing nutrient absorption, and protecting against pathogens, leading to increased yields.	[43,53,69]
Abiotic stress tolerance	Help plants cope with drought and salinity by promoting beneficial root–microbe interactions, improving water uptake, and enhancing root growth.	[8,44,46]
Soil microbial health	Enhance microbial diversity and interactions in the rhizosphere, improving soil health, structure, and long-term fertility.	[41,61,78]
Disease suppression	Biofertilizers containing *Pseudomonas* and *Trichoderma* induce systemic resistance, reducing crop disease incidence and minimizing chemical pesticide use.	[43,65,79,80]
Phosphorus solubilization	Biofertilizers with PSB enhance phosphorus availability, which is critical for crop nutrition and productivity, especially in phosphorus-limited soils.	[12,24]
Climate resilience	Biofertilizers containing climate-resilient microbes can help plants withstand environmental stresses, such as temperature fluctuations and extreme weather.	[16,28,40]
Phytoremediation	Siderophore-producing bacteria and other PGPRs assist in the phytoremediation of heavy metals, contributing to soil detoxification and rehabilitation of polluted lands.	[48,53]
Greenhouse gas reduction	Improved nutrient use efficiency with biofertilizers reduces nitrous oxide (N_2O) emissions, a potent greenhouse gas associated with synthetic fertilizer overuse.	[13,60]
Crop quality enhancement	Biofertilizers improve the nutritional quality of crops (e.g., higher vitamin, protein, and mineral content) by improving nutrient absorption, especially phosphorus and potassium.	[22,81]
Synergy with organic amendments	Biofertilizers work synergistically with organic matter (compost, manure) to enhance microbial activity, nutrient availability, and sustainable nutrient release.	[19,61,80]
Biofertilizers as microbial carriers	Biofertilizers can act as carriers for diverse microbial communities, improving soil microbiome composition and stability, particularly when combined with amendments like biochar.	[30,61]
Marginal climate applications	Cold-tolerant microbial strains in biofertilizers enhance crop performance in cold or marginal soils, improving yield and plant health in difficult growing conditions.	[15]

4.2. Pest Management

There is growing interest in integrating beneficial microbes with PPT to develop sustainable pest management systems. Endophytic fungi in PPT could offer systemic protection against fall armyworm infestations [5,35]. F. Chidawanyika et al. [5] suggested that incorporating endophytic fungi into PPT could provide additional protection against pests like the fall armyworm. I.S. Sobhy et al. [35] examined the effectiveness of PPT in controlling fall armyworms through the use of volatile compounds. Although their study did not focus on endophytic fungi, it provides a solid foundation for considering how endophytes could complement existing pest management strategies in PPT. Other studies have highlighted the potential of endophytic fungi in enhancing plant resistance against pests and diseases [67,73]. These studies emphasize the need for further research to assess the long-term effectiveness of such biological control agents.

Z.R. Khan et al. [7] indicated that specific microbial inoculants significantly boosted plant growth and pest resistance, particularly for controlling stem borers and Striga weed in maize. Nevertheless, the interaction between microbial strains and their combined effects on multiple pest species requires further investigation. If integrated with PPT, PGPR could improve crop growth and pest resistance [39]. However, research gaps include identifying the most effective PGPR strains and understanding their interaction with other components of PPT across various agroecological zones.

D.E. Conlong et al. [4] suggested that entomopathogenic nematodes (EPNs) could significantly reduce stemborer populations and enhance crop yields. Z.R. Khan et al. [34] evaluated the effectiveness of PPT in increasing natural parasitism of maize stemborers. Although their study does not specifically evaluate EPNs, it provides a context in which EPNs could be effectively integrated to control stemborers. Further studies are needed to optimize application methods and assess the long-term sustainability and non-target effects of EPNs in diverse farming systems. C.A. Midega et al. [76] found that AMF inoculation improved plant growth and nutrient uptake, thereby increasing resilience to pest attacks. However, more detailed research is required to identify the specific AMF strains that offer the best protection and to understand how they interact with other soil microorganisms and agricultural practices. M. Dong et al. [82] found that microbial inoculants enhanced the production of volatile organic compounds (VOCs), which attracted natural enemies of pests and improved pest control efficacy. Similarly, I.S. Sobhy et al. [35] highlighted how PPT companion crops, when combined with microbial inoculants, produce bioactive VOCs that repel pests, such as the fall armyworm, and attract natural enemies, like parasitoids, enhancing pest control. Future research should focus on identifying which microbial inoculants are most effective at enhancing VOC production and how environmental factors influence VOC release and pest attraction. While these studies underscore the potential benefits of integrating beneficial microbes with PPT, several research gaps remain. These include understanding the specific interactions and synergies between different microbes, optimizing inoculation methods, assessing long-term sustainability, and adapting these systems to diverse environmental conditions and crop varieties. Addressing these gaps will be key to fully harnessing the potential of microbial inoculants in enhancing the efficacy and sustainability of push–pull technology in pest and soil fertility management.

4.3. Disease Suppression

Several studies have consistently demonstrated that biofertilizers play a significant role in suppressing soil-borne pathogens through various mechanisms, including the production of microbial antagonistic compounds, direct competition with pathogens, and the induction of systemic resistance in plants [2,65,73,79,82]. Biofertilizers have also been shown to enhance soil microbial diversity, supporting disease suppression and promoting sustainable disease management [82]. For example, biofertilizers effectively controlled Panama disease in banana cultivation by promoting beneficial microbial communities that outcompete the pathogenic fungus *Fusarium oxysporum* [79]. M. Dong et al. [82] explored the role of biofertilizers in suppressing bacterial wilt in tomatoes, demonstrating how

biofertilizer application alters microbial communities in soil microaggregates to combat the disease. Similarly, applying plant PGPR in mustard reduced the severity of Alternaria blight through the induction of systemic resistance and the production of pathogen-inhibiting secondary metabolites [64]. Additionally, *Trichoderma virens*, a well-known biofertilizer, has proven effective in controlling soil-borne diseases and enhancing crop resilience by promoting root colonization and producing lytic enzymes that degrade pathogen cell walls [65]. Z. Shen et al. [80] demonstrated how compost and biofertilizers can suppress Fusarium wilt in bananas, enhancing yield and quality while modifying the soil microbiome to resist pathogen proliferation. C. Tao et al. [73] investigated fungal interactions within biofertilizers and their combined effects in suppressing Fusarium wilt, revealing the additive effects of fungal components in promoting plant growth while controlling disease. The synergistic interaction of multiple fungal species in biofertilizers further underscores the effectiveness of diverse microbial consortia for disease suppression, offering better results than single-strain treatments [73]. These findings highlight the importance of utilizing diverse biofertilizer formulations for comprehensive disease management in agricultural systems, which can be integrated with PPT and ensure sustainable agroecosystems.

4.4. Soil Health

Biofertilizers have been applied to promote beneficial microbes, enhancing the availability and uptake of essential soil minerals, thereby contributing to the overall success of push–pull technologies in sustainable agriculture. One of soil microbes' most important agroecosystem services is improving nutrient bioavailability. For instance, plant roots cannot absorb insoluble phosphate, potassium salts, or certain forms of phosphorus and potassium in the soil [83,84]. Nitrogen-fixing microbes utilize the enzyme nitrogenase to convert atmospheric nitrogen into forms like NH_4^+ or NO_3^-, which plant roots can readily absorb [83]. Although organic phosphorus compounds (such as phosphoinositides, phospholipids, and nucleic acids) and inorganic sources (like phosphate rock and insoluble phosphate) dominate in the soil, they are not directly accessible to plants.

Research has demonstrated various benefits. C.A. Midega et al. [76] found that nitrogen-fixing bacteria in PPT improved soil nitrogen levels and plant health, leading to better pest resistance and higher yields. Rhizobium can symbiotically form root nodules with the roots of legume crops and fix nitrogen efficiently in these nodules [15]. Azospirillum, however, is less effective in fixing nitrogen than the symbiotic fixation in legume nodules but can fix nitrogen in non-legume crops such as maize, rice, and wheat [85]. B. Vanlauwe et al. [19] showed that phosphate-solubilizing bacteria in legume-based cropping systems enhanced phosphorus availability and uptake, improving plant vigor and reducing pest damage. Similarly, W. Janati et al. [11] reported that phosphate-solubilizing bacteria in maize cropping systems significantly increased phosphorus solubility, improving phosphorus uptake by maize plants and enhancing pest resistance.

P.N. Bhattacharyya et al. [69] found that zinc-solubilizing bacteria increased zinc solubility, enhancing zinc uptake by maize plants and improving plant health and pest resistance. H. Etesami et al. [12] reviewed the role of potassium-solubilizing bacteria in biofertilizers, which improve soil health by making essential nutrients more accessible to plants and increasing overall soil fertility. A.A. Mahmud et al. [1] reported that silicon-solubilizing bacteria increased silicon uptake in rice plants, improving their structural integrity and resistance to pest attacks. Z.R. Khan et al. [7] indicated that certain microbial inoculants significantly boosted plant growth and pest resistance by improving nutrient uptake, particularly nitrogen and phosphorus. J. Nelkner et al. [57] examined the effects of long-term farming practices on soil microbiomes using metagenomically assembled genomes (MAGs), finding that biofertilizer applications in these systems promoted beneficial microbes that play key roles in enhancing nutrient availability and soil health. D. Francioli et al. [61] compared mineral and organic amendments, including biofertilizers, and found that biofertilizers significantly improved soil microbial activity and community structure. This increase in agriculturally relevant microbes was directly linked to improved

soil health and fertility. S. Bolan et al. [30] reviewed the potential of biochar as a microbial carrier in biofertilizers, finding that biochar improves the efficiency of biofertilizers by providing a habitat for beneficial microbes, thereby enhancing soil fertility and health over the long term. These comprehensive findings highlight the benefits of various biofertilizers in enhancing nutrient availability and uptake. However, further research is needed to better understand the specific interactions and synergies between different biofertilizers and their combined effects on plant health and pest resistance in PPT across diverse agroecosystems.

4.5. Water Management

Several studies have demonstrated the potential of beneficial microbes in crop production, which can be integrated with PPT to improve drought resilience, water use efficiency, and crop yields across various cropping systems. AMF inoculation has been shown to enhance root water absorption, increasing drought resilience and crop yields in water-limited environments [51,62], which could benefit rainfed PPT in semi-arid regions. When combined with optimized irrigation schedules, nitrogen-fixing bacteria improved water use efficiency and crop performance under varying water availability conditions [13,19]. J. Li et al. [28] observed that microbial inoculants enhanced wheat growth under low soil water availability, leading to more efficient water use and improved crop resilience during dry spells. Microbial inoculants in biofertilizers improve wheat's drought tolerance by colonizing the root zone and enhancing soil water retention. M. Rafique et al. [29] demonstrated that integrating biochar and microbial inoculants improved soil water retention capacity and overall crop yield under fluctuating rainfall conditions. O.A. Fasusi et al. [70] emphasized that biofertilizers improve rhizosphere management, enhancing the soil's ability to retain water and resist drought conditions. A.A. Mahmud et al. [1] discussed how biochar and microbial inoculants work together to improve soil structure and water retention capacity. These improvements in soil conditions contribute to higher crop yields, particularly in areas with variable rainfall. Additionally, S. Bolan et al. [30] reviewed the role of biochar-enhanced biofertilizers in improving water retention in soils. Biochar acts as a microbial carrier, improving the ability of soils to retain moisture, especially in dry or degraded soils. P.N. Bhattacharyya et al. [69] reviewed how water-efficient irrigation techniques combined with beneficial microbes in rice fields resulted in significant water savings, improved plant water status, and enhanced pest control efficacy. S. Compant et al. [40] examined how climate change affects plant–microbe interactions, noting that biofertilizers can mitigate the impacts of water stress by improving plant water-use efficiency and promoting microbial communities that help retain soil moisture. Integrating cover crops, beneficial microbes, and optimized water management practices improved soil moisture dynamics and crop productivity, enhancing soil moisture retention, reducing irrigation requirements, and improving overall crop performance [56,61]. However, more research is needed to better understand the specific interactions and synergies between microbial inoculants and their combined effects on water use efficiency and crop resilience across different cropping systems and environmental conditions.

5. Microbial Mechanisms for Sustainable Crop Production in Challenging Soils

Several studies show that biofertilizers consistently increase microbial diversity, especially in challenging soils such as those that are arid, saline, alkaline, or polluted [86–90]. F. Zhang et al. [87] explored the application of the fungal biofertilizer *Trichoderma* in saline and alkaline soils. *Trichoderma* improved plant growth and soil nutrient availability by altering the soil microbiome to favor salt-tolerant and pH-resilient microbial populations. The application of *Trichoderma* increased the abundance of *Firmicutes* and *Proteobacteria*, two phyla that are well known for their tolerance to saline and alkaline conditions. Specifically, salt-tolerant species, such as *Bacillus* (Firmicutes), were promoted. Enhanced microbial diversity was observed, with a higher proportion of bacteria involved in nutrient solubilization, particularly phosphorus, which is often limited in saline–alkaline soils.

X. Wei et al. [88] combined the use of *Bacillus* and microalgae biofertilizers, significantly improving the growth and biomass of *Salvia miltiorrhiza* by altering soil microbial communities. The study found an increase in *Firmicutes* (due to introducing *Bacillus* strains) and *Cyanobacteria*, known to enhance N fixation and nutrient availability in nutrient-poor or stressed environments. Introducing biofertilizers promoted microbial biodiversity, mainly by enriching beneficial nitrogen-fixing and phosphorus-solubilizing microorganisms. Additionally, N. Sivaprakasam et al. [90] used metagenomics to assess the dynamics of PGPMs in soils where pathogens like *Phytophthora* are prevalent. Biofertilizer application increased *Firmicutes* and *Proteobacteria* and especially beneficial PGPMs, such as *Bacillus* (Firmicutes) and *Pseudomonas* (Proteobacteria). These bacteria enhance plant growth by producing enzymes and compounds that suppress pathogens and improve nutrient availability. The application of biofertilizers enriched microbial diversity, promoting beneficial microbial communities involved in disease suppression and nutrient cycling, particularly in soils with poor health or pathogen issues. M. Mącik et al. [89] emphasized the role of phosphorus biofertilizers in regenerating soils suffering from nutrient deficiencies, including those in arid regions. The application of phosphorus biofertilizers led to an increase in *Actinobacteria*, a phylum well-adapted to nutrient-poor and dry soils, and *Proteobacteria*, which are crucial for phosphorus solubilization. These microbial changes significantly improved nutrient cycling and soil health, enhancing microbial diversity and particularly favoring microbial communities involved in phosphorus solubilization and drought resilience.

Sun et al. [86] assessed the application of *Bacillus subtilis* biofertilizer in agricultural soils with high ammonia emissions, a problem often exacerbated by poor management or pollution. The application led to a shift in soil microbiomes, with an increase in *Firmicutes* (particularly *Bacillus subtilis*), which are known for their role in reducing ammonia emissions through enhanced nitrogen cycling. *Nitrospirae*, crucial for nitrification, also increased. The biofertilizer enhanced overall microbial diversity, significantly increasing functional microbes in N cycling, reducing ammonia loss, and promoting healthier soil conditions. These studies demonstrate that biofertilizers promote the growth of resilient phyla such as *Firmicutes, Proteobacteria*, *Nitrospirae*, and *Actinobacteria* in extreme soils. These bacterial groups are essential for nutrient cycling, stress tolerance, and soil remediation. Additionally, biofertilizer applications lead to increased microbial diversity in arid, saline, alkaline, and polluted soils. This diversity is crucial for improving soil functionality, promoting plant growth, and enhancing ecosystem resilience under stress. Furthermore, in challenging soil environments, biofertilizers enhance the presence of functional microbial groups involved in nutrient solubilization (e.g., phosphorus and nitrogen), salt tolerance, and pollutant degradation, all of which contribute to the restoration of soil health.

6. Biofertilizer-Mediated Changes in Microbial Diversity

Several studies have reported mixed results regarding the effects of biofertilizers on microbial diversity and ecosystem functioning [9,78,91]. A.A. Jalloh et al. [9] highlighted that biofertilizers increased microbial diversity, including beneficial bacterial and fungal genera, which enhanced soil resilience and overall crop performance in farmers' fields. Although beneficial microorganisms thrived and contributed to improved ecosystem functions, such as nutrient availability, there were instances where microbial diversity did not increase uniformly. This variability suggests that the success of biofertilizers may depend on bacterial phyla.

A.A. Otaiku et al. [91] demonstrated that biofertilizers enhanced soil microbiome diversity by introducing beneficial microorganisms, which improved soil health and nutrient cycling in a cassava system. The application of *Bacillus subtilis* biofertilizer increased the relative abundance of *Proteobacteria* and *Firmicutes*, particularly *Bacillus* spp., and also increased the abundance of *Nitrospirae*, responsible for ammonia oxidation [86]. This shift suggests improved N cycling and reduced ammonia loss, where biofertilizers enhanced overall microbial biodiversity, supporting functional groups that improved N retention and soil health. M. Mącik et al. [89] reported a marked increase in *Actinobacteria*, which

play a critical role in P solubilization. *Firmicutes* and *Proteobacteria* also increased, while the abundance of *Bacteroidetes* decreased, indicating improved P cycling, nutrient availability, and enhanced soil fertility. The application of *Trichoderma* biofertilizers in *Medicago sativa* (alfalfa) cultivation in alkaline–saline soils led to changes in microbial diversity, particularly favoring bacteria capable of surviving in stressed environments [87]. *Firmicutes* and *Proteobacteria* showed an increase, indicating enhanced tolerance to saline–alkaline conditions, while *Bacteroidetes* and *Acidobacteria* exhibited shifts that were context specific, depending on soil salinity and pH levels.

In another study, X. Wei et al. [88] observed an increase in *Cyanobacteria*, Actinobacteria, and *Firmicutes*. *Bacillus* strains dominated the bacterial community, with a noticeable shift in N-fixing and P-mobilizing bacterial phyla. N. Sivaprakasam et al. [90] showed a rise in *Firmicutes* and *Proteobacteria*, particularly those linked to pathogen suppression, such as *Bacillus* spp. A decrease in *Bacteroidetes* and *Chloroflexi* was observed, possibly due to competitive exclusion by beneficial PGPMs. D. Agustiyani et al. [27] explored the biofertilizer potential of PGPR from different plant ecosystems. They identified shifts in dominant bacterial phyla such as *Proteobacteria*, *Firmicutes*, and *Actinobacteria* across various ecosystems. The introduction of PGPR increased the abundance of beneficial bacterial groups, which promoted plant growth and altered microbial biodiversity. M. Dong et al. [82] investigated biofertilizer applications in relation to soil microbial assembly. They found that biofertilizers triggered a shift in microbial communities, particularly increasing the abundance of *Proteobacteria*, *Acidobacteria*, and *Firmicutes*, which are often linked to disease suppression. This microbial shift contributed to the suppression of bacterial wilt in tomato plants. D. Francioli et al. [61] studied the impact of long-term fertilization strategies (mineral vs. organic amendments) on microbial communities. Their findings demonstrated that organic amendments significantly increased microbial diversity, particularly in the phyla *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. Organic inputs fostered a more diverse and functionally active microbial community than mineral fertilizers. P.P. Bittencourt et al. [8] examined the role of bacterial inoculants under drought stress. They observed that inoculants, including species from the *Bacillus* and *Pseudomonas* genera, led to significant changes in microbial biodiversity. There was an increase in the abundance of stress-tolerant bacterial phyla, such as *Firmicutes* and *Actinobacteria*, enhancing plant drought tolerance. These studies show that biofertilizer application often shifts bacterial community composition, with increased abundance in beneficial phyla like *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Nitrospirae* while often suppressing harmful or less beneficial bacterial groups. These shifts contribute to improved soil health, plant growth, and ecosystem functioning.

7. Future Directions and Research Needs

Several key areas of research require attention to advance the application of biofertilizers and enhance sustainable agricultural practices. One priority is the integration of biofertilizers with agroecological systems like PPT. This involves optimizing the synergy between biofertilizers, companion crops, and microbial inoculants to improve pest management, soil fertility, and overall system sustainability. Additionally, developing reliable and scalable methods for biofertilizer production and application is essential. Research should focus on refining biofertilizer formulations, delivery mechanisms, and compatibility with various crop varieties and soil conditions to facilitate broader farmer adoption. Despite the potential of biofertilizers to enhance soil health and crop productivity, their performance remains variable and unpredictable. To address this, research must explore improving their consistency and effectiveness across different environmental conditions and soil types. This requires a deeper understanding of microbial interactions within the plant–soil continuum and the specific roles of microbial consortia in nutrient cycling, disease suppression, and stress tolerance, particularly within PPT systems. As climate change increasingly impacts agricultural systems, biofertilizers could be vital in boosting crop resilience to environmental stresses such as drought, salinity, and extreme weather events. Research

should investigate how biofertilizers enhance soil water retention and plant tolerance to these abiotic stresses, which will be critical for developing sustainable cropping systems in vulnerable regions. While short-term studies have shown the benefits of biofertilizers, long-term field trials are necessary to assess their effects on crop yields, soil health, and ecosystem services over time. This will provide a more comprehensive understanding of the sustainability of biofertilizer use across diverse agroecosystems.

8. Conclusions

Applying biofertilizers in agroecological systems, particularly in conjunction with PPT, has significant potential to advance sustainable agricultural practices. This review highlights the ability of biofertilizers to enhance nutrient availability, improve soil health, and boost crop productivity by fostering beneficial plant–microbe interactions. In addition, biofertilizers play a critical role in improving pest management and crop resilience, especially under environmental stress conditions like drought. However, the practical implementation of biofertilizers faces challenges such as variability in performance, complex interactions within the soil–plant–microbe continuum, and inconsistent outcomes across different agroecological contexts. Addressing these challenges will require refining biofertilizer formulations, improving production methods, and optimizing application techniques. This review also demonstrates that biofertilizers can significantly reduce farmer's reliance on synthetic inputs, contributing to more sustainable and resilient cropping systems. Therefore, farmers can better integrate these tools into their practices to improve crop yields and support ecosystem services. However, a multidisciplinary approach involving agronomists, microbiologists, and farmers is crucial to advancing biofertilizer technology for successful adoption. Long-term studies and field trials are essential to validate the benefits of biofertilizers in diverse environments and ensure their scalability for widespread use. Ultimately, biofertilizers offer a promising path toward enhancing sustainable agriculture, improving food security, and mitigating the environmental impacts of modern farming practices.

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New Insights on the Glyphosate-Degrading Enzymes C-P Lyase and Glyphosate Oxidoreductase Based on Bioinformatics

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Abstract: Bioremediation, the degradation of environmental pollutants by living organisms, has immense potential to lead to a greener planet. Bioinformatics analysis can contribute to the identification of novel microorganisms, which biodegrade contaminants, or of participating proteins and enzymes, and the elucidation of the complex metabolic pathways involved. In this study, we focus on C-P lyase and glyphosate oxidoreductase (Gox), two enzymes which degrade glyphosate, a widely used pesticide. Amino acid sequences of the two enzymes were collected from a broad range of microorganisms using the KEGG database and BLAST. Based on this, we identified additional lineages, with putative glyphosate-degrading activity, for which no glyphosate-degrading species have been reported yet. The conserved residues in each enzyme were identified via multiple alignments and mapped onto the 3D structures of the enzymes, using PyMOL, leading to novel insights into their function. As the experimental structure of Gox is still unknown, we created structural models based on three different programs and compared the results. This approach can be used to yield insights into the characteristics of potential glyphosate-degrading enzymes. Given the limited information available, such a step is important to gain further knowledge about them, which can contribute to their application in bioremediation in the future.

Keywords: glyphosate; C-P lyase; glyphosate oxidoreductase; biodegradation; bioinformatics; phylogenetics

1. Introduction

Glyphosate, *N*-(phosphonomethyl)glycine, is an active ingredient in many widely used, broad-spectrum herbicides (e.g., Roundup^{TM}). It is a synthetic amino acid analogue of glycine incorporating a phosphonic acid [1]. It inhibits enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), resulting in the inhibition of the shikimate pathway, which is essential for the biosynthesis of aromatic amino acids in plants [2].

Glyphosate has been characterized as a safe compound by the European Food Safety Authority (EFSA) for human health, animals, and the environment, as "no critical areas of concern" were confirmed. Additionally, the European Chemicals Agency (ECHA) does not classify glyphosate as carcinogenic or possibly carcinogenic [3]. On the other hand, it is considered as "probably carcinogenic" to humans (Group 2A) according to the International Agency for Research on Cancer (IARC) [1] and it is labeled as "toxic to aquatic life with long lasting effects" as per the Hazard Statements of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) [1]. Glyphosate was an approved pesticide until December 2023 in the E.U. [3], where its use was recently extended for 10 more years [4].

Nevertheless, there is increasing concern about the accumulation of pesticide residues and their metabolites in soils and food crops. In soil sampled from agricultural sites throughout the E.U. and other cooperating countries, glyphosate and its metabolite alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were among the most frequently detected substances and also among those with the highest concentrations [5].

A case study in central Germany yielded similar results, as glyphosate and AMPA were detected at the highest concentrations in surface water and topsoil samples [6]. Concerns have also been raised about the persistence of glyphosate and its metabolite AMPA in soil, especially in fields where glyphosate-based herbicides have been extensively used [3,7], its potential to bioaccumulate in terrestrial organisms [8], and its impact on crop health [3], while it has also led to glyphosate-resistant weeds [9]. Both compounds are transported off site with rainfall, contaminating surface water, hedgerows, and adjacent non-treated fields [7,8].

In particular, glyphosate can affect the microbial population, and hence, have an impact on plant-beneficial microbes [10] and cause metabolic changes in plants, even at low doses [11]. Glyphosate and its salt form, isopropylamine (IPA), also act as chelating agents by binding to metal cations necessary for plant nutrition [12]. Additionally, studies have shown potential negative effects on insects; i.e., rendering insects more prone to infections, inhibiting the production of melanin, disturbing their gut microbiome [13–16], and leading to a low responsiveness to sucrose [17] and impaired aversive learning [18]. Aquatic organisms are also affected: in zebrafish and other commercial fish, such as Nile tilapia, glyphosate has been linked to developmental problems [19], cardiovascular toxicity [20], oxidative stress and anxiety [21], and kidney disfunction [22]. Glyphosate impacts aquatic ecosystems via changes in the abundance and composition of freshwater picoplanktonic populations [23] and certain species of marine phytoplankton [24]. Glyphosate can have a negative impact on mammals by altering gene expression and affecting DNA methylation in human cells [25–27]. The glyphosate-based herbicide Roundup^{TM}, glyphosate, and AMPA can indirectly cause DNA damage by inducing reactive oxygen species (ROS) production in human peripheral blood mononuclear cells [28]. In addition, exposure of human lymphocytes to glyphosate in higher concentrations (200 μmol/L) than those normally found in the environment (20–40 μmol/L) demonstrated genotoxic effects [29]. It has also been associated with increased urinary glyphosate and AMPA levels in humans [30], which, in turn, have been associated with longer telomere length in leukocytes [31]. Low chronic maternal exposure to glyphosate has also been associated with reduced immune response in mice [32].

The degradation of glyphosate can be either biotic, by microorganisms in both aerobic and anaerobic conditions, or abiotic, by Mn-containing minerals [33], and depends on many factors, e.g., whether it is found in water or soil, soil properties, weather conditions, and cultivated plants in the area. Regarding its degradation rate, half-life estimates range from 5 to 180 days [34].

There is limited current knowledge about the pathways involved in phosphonate degradation, including glyphosate, their regulation, and the relevant enzymes [35]. Three enzymes have been reported to degrade glyphosate: C-P lyase, which cleaves the bond after phosphorus; and glyphosate oxidoreductase (Gox) and glycine oxidase (GO), which both cleave the C-N bond. The last two enzymes produce the same products but their function is based on a different mechanism (Figure 1).

C-P lyase is a protein complex that consists of two copies each of four proteins (PhnG, PhnH, PhnI, PhnJ) and one ATP-binding subunit, PhnK. The degradation of glyphosate by C-P lyase, which takes place under inorganic phosphate (Pi) deficiency [35], results in the production of sarcosine and phosphate. The C-P lyase complex is part of the C-P lyase pathway, which converts phosphonate into PRPP (5-phosphoribosyl-a-1-diphosphate) using ATP [35]. According to the literature, it is widely present across bacteria, and shows broad substrate specificity, as it cleaves the C-P bond of organophosphonates [36]. At first, the tertiary structure of the complex was determined without PhnK (PDB accession number: 4XB6) [37]. A few years later, PhnK was also included in the complex via single-particle cryogenic electron microscopy, which provided valuable insights into the interactions of PhnK with the core complex and the conformational changes before and after utilizing ATP (PDB accession number: 7Z19, with ATP: 7Z18), and also, with PhnL in various conformations (PhnK/PhnL dual ABC dimer, PDB accession numbers: 7Z15, 7Z16) [38].

PhnJ is the active site of the enzyme (Figure S1). It contains an Fe_4S_4 complex and a Zn molecule, which is stabilized by three conserved Cys residues (Cys241, Cys244, and Cys266), found within the conserved motif CX2CX21C. Two more residues are important for the reaction mechanism of the complex: Gly32 and Cys272 [39]. Due to the presence of the fourth conserved Cys residue, it can be included as part of the aforementioned motif, namely, as CX2CX21CX5C [39]. Cys272 is responsible for the generation of a thiyl radical, which cleaves the C-P bond of 5-phosphoribosyl-1-phosphonate. Notably, PhnJ does not contain the signature radical SAM enzyme motif (CX3CX2C), which stabilizes the Fe_4S_4 complex [39]. A second potential active site is located between PhnJ and PhnI, which includes the residues His328 and His333 (PhnI) and His108 (PhnJ). Four further domains (Figure S1) have been described [37]:

- CMD: C-terminal mini domain (PhnJ);
- CID: Central insertion domain (PhnJ), which could participate in the binding of PhnK to PhnJ;
- BBD: Beta barrel domain (PhnI);
- NTD: N-terminal domain (PhnI).

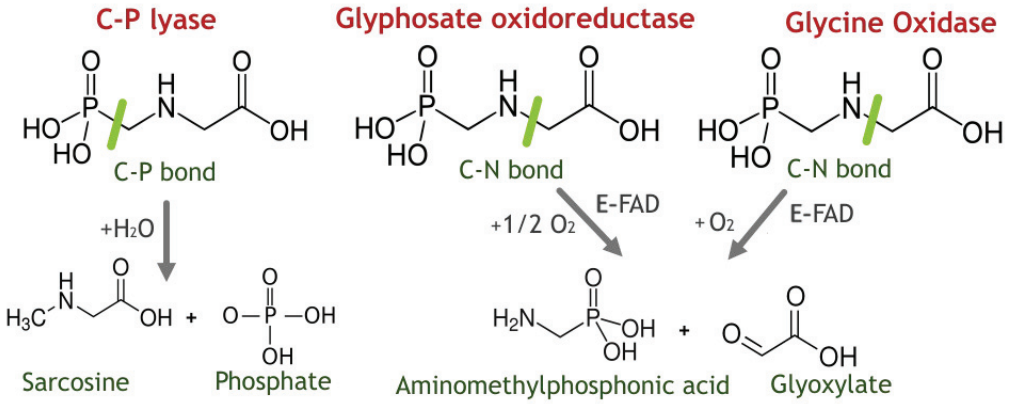

Figure 1. The enzymes degrading glyphosate, their reaction mechanisms, and the resulting products. Glyphosate image from https://en.m.wikipedia.org/wiki/File:Glyphosate.svg (accessed on 28 November 2021).

It has been proposed that not all C-P lyases are specific to glyphosate, and that C-P lyase of *Eschericha coli* is an example of an (amino)alkylphosphate-specific enzyme without glyphosate-degrading activity [35,40]. For this reason, there is the hypothesis that two different types of independently induced C-P lyases exist [35]. However, later research on *Ochrobactrum anthropi* and *Achromobacter* sp. [41] could not provide evidence for the hypothesis of two distinct C-P lyases (Sviridov A, personal communication).

The glyphosate oxidoreductase (Gox) gene was first characterized from *Achromobacter* sp. LBAA, a bacterial strain isolated in a glyphosate waste treatment facility, as part of a Monsanto patent [42], in order to construct glyphosate-resistant plants. Gox is a monomeric FAD-dependent enzyme, cleaving the C-N bond of glyphosate and yielding glyoxylate and the metabolite AMPA. It is speculated that the reduction of FAD takes place in the active site of the enzyme by glyphosate [42]. There is no known experimental structure of Gox, although a computed structural model is available [43].

Glycine oxidase (GO) is the third enzyme known to degrade glyphosate. Several GO structures are available in the PDB (e.g., 3IF9, 1NG4 and 1RYI from *Bacillus subtilis*). It is a homotetrameric enzyme and its substrates include glycine, neutral D-amino acids, and sarcosine. Similar to Gox, it is an FAD-dependent enzyme and it cleaves the C-N bond, yielding the same degradation products: AMPA and glyoxylate. However, Gox and GO show low sequence identity [2] and, based on oxygen utilization, appear to have a different reaction mechanism: oxygen reduction in Gox does not result in the production

of hydrogen peroxide, which is the case for GO [2,42]. Several studies have focused on engineering GO in order to enhance its glyphosate-degrading activity [2,44–47].

Given the negative consequences of glyphosate, in this study we look at the characteristics of the proteins of the glyphosate-degrading enzymes at a sequence level as a way to understand and optimize their function, by utilizing bioinformatics tools. As GO has been extensively studied and improved through mutagenesis, we focus on C-P lyase and Gox. Further insights into the enzymatic degradation of glyphosate by bacteria can contribute to the eventual application of bioremediation techniques for a faster removal of glyphosate from affected areas.

2. Materials and Methods

Information on the bacterial species reported to degrade glyphosate and the pathways or enzymes involved in this activity was collected from relevant references in PubMed [48] and ScienceDirect [49] (Table 1). These databases, along with PDB [50], were also used to find the already-available information on enzymes responsible for glyphosate degradation, namely, Gox, C-P lyase, and GO.

For C-P lyase, the amino acid sequences for each subunit of the complex were collected using the KEGG Orthology (KO) database [51] and the following entries: PhnJ: K06163, PhnI: K06164, PhnH: K06165, PhnG: K06166, and PhnK: K05781. The available sequences were parsed in such a way that a wide variety of representatives from across the bacterial kingdom were included; the selection was performed manually, using the taxonomy option of the KO entry and selecting at least one representative per family. The selected amino acid sequences are available in the Supplementary Dataset in folder "C-P lyase". The sequence selected from each taxonomical group was random and we also included the sequence of *E. coli*. MUSCLE [52] was used for the multiple alignments, and the highly conserved residues were marked onto the 3D structure using PyMOL (linux version 2.5.2) [53]. The absolutely conserved residues were selected based on Jalview [54] (scoring 11); residues in positions with a Jalview score of 10 were also selected, which reflects that, despite variation, physicochemical properties are conserved [54]. The ScanProsite tool (https://prosite.expasy.org/scanprosite/, accessed on 15 March 2022) was used to search for motifs in the PhnJ sequences of the C-P lyase complex.

As there is no KEGG Orthology (KO) group for Gox, BLASTp [55] searches for orthologs in other species were performed using as a query the Gox amino acid sequence of *Ochrobactrum* sp. G-1 (ACZ58378.1). Two different databases were used:

- The nr database (BLASTp_nr), which includes all non-redundant GenBank CDS translations and also entries from PDB, SwissProt, PIR, and PRF, excluding environmental samples from WGS projects.
- RefSeq (BLASTp_ref), which contains only curated entries, i.e., one representative or "Select" transcript for every protein-coding gene, based on specific criteria [55].

Two different BLASTp searches were performed, with default settings, for each database (Table S1), one without any filters (BLASTp_nr_NOGROUP, BLASTp_ref_NOGROUP) and one for each bacterial group (BLASTp_nr_GROUP, BLASTp_ref_GROUP), as defined by Jun et al. [56], aiming at better sampling of more bacterial representatives. The selection of the resulting sequences for BLASTp_nr_NOGROUP and BLASTp_ref_NOGROUP was based on the first 100 sequences with the lowest E-value for each protein query. For the BLASTp_nr_NOGROUP, the highest E-value was 3×10^{-128} and for the BLASTp_ref_NOGROUP 1×10^{-127} (available in Supplementary Dataset). For BLASTp_nr_GROUP and BLASTp_ref_GROUP, the results with the lowest E-value for each protein query from each bacterial group were selected. MUSCLE [52] was used for the multiple alignments.

The Gox 3D structure was computed using I-TASSER [57] and Phyre2 [58], which are based on homology modeling, as well as AlphaFold Colab, which performs ab initio prediction [59]. The default settings were used. A general comparison of the results was made by aligning the structures with PyMOL. The results were assessed using QMEANDisCo, which is part of SWISS-MODEL [60].

3. Results and Discussion

3.1. Distribution of Bacterial Species Previously Reported to Degrade Glyphosate

In the literature, 42 different bacterial species have been reported to degrade glyphosate (Table 1). The majority of the species belong to α-, β-, and γ-proteobacteria, a smaller number to bacilli and actinobacteria, and some to bacteroidetes (Figure 2). The enzymes or genes involved in glyphosate degradation have been identified only in some research papers. In most cases, the possible mechanism was inferred based on the products resulting after glyphosate consumption by the bacteria, specifically, based on the presence of sarcosine (indicating C-P lyase activity) or AMPA (indicating Gox activity). In addition to the three known glyphosate-degrading proteins, the presence of a class II EPSPS synthase-encoding gene (*aroA* gene) has also been reported, although the experimental structure of the protein is not yet known and no further experiments were carried out to test and validate the activity of *aroA* [61]. Consequently, the knowledge about the genes and the structure or mechanism of the enzymes involved in glyphosate degradation is still limited.

Table 1. Bacterial species reported to degrade glyphosate. Depending on the evidence of glyphosate degradation in each paper, "G" represents the discovery of the responsible gene, "P" the presence of the degradation product, and hyphen (-) a decrease in the amount of glyphosate.

Group	Genus, Species	Evidence	Products/Genes Detected	Reference
actinobacteria	*Arthrobacter* sp. GLP-1	P	Sarcosine	[62]
actinobacteria	*Streptomycete* sp. StC	P	Sarcosine	[63]
actinobacteria	*Streptomyces* sp. StA	P	Sarcosine	[63]
actinobacteria	*Rhodococcus soli* G41	G	*soxB* gene	[64]
actinomycetes	*Arthrobacter atrocyaneus* ATCC 13752	P	AMPA	[65]
bacilli	*Geobacillus caldoxylosilyticus* T20	P	AMPA	[66]
bacilli	*Bacillus cereus* CB4	P	AMPA, glyoxylate, sarcosine, formaldehyde	[67]
bacilli	*Lysinibacillus sphaericus*	P	Free phosphorus concentration, glycine	[68]
bacilli	*Bacillus aryabhattai* FACU	G	*goxB* gene	[69]
bacilli	*Bacillus. cereus* 6 P	-	Conversion of glyphosate to polyphosphate	[70]
bacteroidetes	*Flavobacterium* sp. GD1	P	AMPA	[33]
bacteroidetes	*Chryseobacterium* sp. Y16C	P	AMPA	[71]
αctinobacteria	*Arthrobacter atrocyaneus* ATCC 13752	P	AMPA	[65]
α-proteobacteria	*Agrobacterium radiobacter*	P	AMPA	[33]
α-proteobacteria	*Ochrobactrum anthropi* GPK 3	P	AMPA	[72]
α-proteobacteria	*Ochrobactrum* sp. GDOS	P	AMPA	[73]
α-proteobacteria	*Ochrobactrum* sp. G1	G	*gox* gene	[42]
α-proteobacteria	*Agrobacterium radiobacter* SW9	P	AMPA	[74]
α-proteobacteria	*Rhizobiaceae meliloti* 1021	G	Homology to part of the *phn* gene cluster of *E. coli*	[75]
α-proteobacteria	*Ochrobactrum* sp. GDOS	P	AMPA	[73]
α-proteobacteria	*Agrobacterium tumefaciens* CHLDO	G	*phn* gene cluster	[76]
α-proteobacteria	*Ochrobactrum haematophilum* SR	-	-	
α-proteobacteria	*Ochrobactrum intermedium* Sq20	G, P	Sarcosine, glycine, *aroA* gene (class II EPSPS)	[61]
β-proteobacteria	*Comamonas odontotermitis* P2	G	*gox* and *phnJ* genes	[77]
β-proteobacteria	*Achromobacter* sp. strain MPK 7A	P	Sarcosine	[72]
β-proteobacteria	*Achromobacter* sp. Kg 16	P	AcGP(*N*-acetylglyphosate), possibly AMPA	[72]
β-proteobacteria	*Achromobacter* Group V D (*Agrobacterium* sp. LW9)	P	AMPA	[74]

Table 1. *Cont.*

Group	Genus, Species	Evidence	Products/Genes Detected	Reference
β-proteobacteria	*Achromobacter* sp. MPS 12A	P	Methane, sarcosine, glycine	[41]
β-proteobacteria	*Alcaligenes* sp. GL	P	Sarcosine	[78]
β-proteobacteria	*Burkholderia vietnamiensis* strain AQ5-12	-	-	[79]
β-proteobacteria	*Achromobacter denitrificans* SOS5	-	-	[76]
β-proteobacteria	*Achromobacter insolitus* SOR2	-	-	[76]
β-proteobacteria	*Achromobacter xylosoxidans* SOS3	-	-	[76]
β-proteobacteria	*Achromobacter insolitus* str Kg 19 (VKM B-3295)	G	*phnJ* gene	[80]
γ-proteobacteria	*Pseudomonas* sp. LBr	P	AMPA, a very small amount of glycine 5%	[81]
γ-proteobacteria	*Providencia rettgeri* GDB 1	P	AMPA	[82]
γ-proteobacteria	*Pseudomonas* sp. 4ASW	P	Sarcosine	[83]
γ-proteobacteria	*Enterobacter cloacae* K7	P	Sarcosine, glycine	[84]
γ-proteobacteria	*Enterobacter* sp. Bisph2	-	-	[85]
γ-proteobacteria	*Pseudomonas pseudomallei* 22	G, P	Phosphotransferase genes *glpA* and *glpB*	[86]
γ-proteobacteria	*Pseudomonas* sp. GLC11	-	-	[87]
γ-proteobacteria	*Pseudomonas* sp. PG2982	P	Sarcosine	[88]
γ-proteobacteria	*Pseudomonas* sp. SG-1		Glyphosate concentration decreased	[89]
γ-proteobacteria	*Pseudomonas nitroreducens* TR3	-	-	[76]

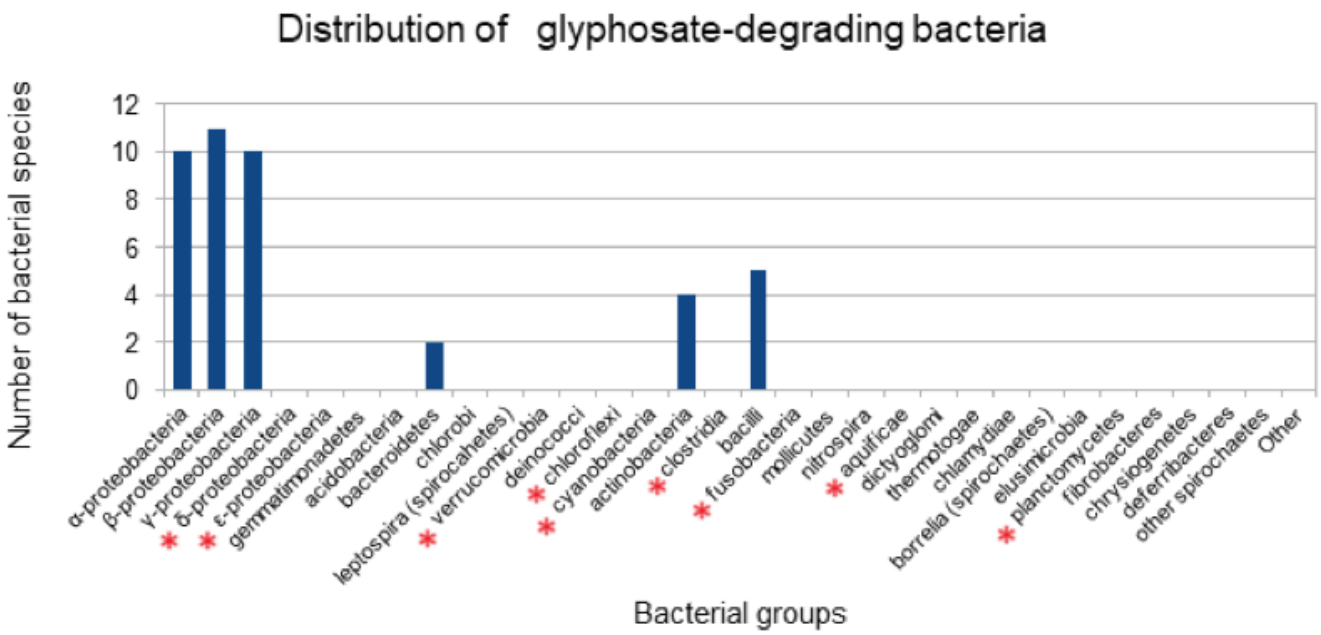

Figure 2. Distribution of the bacterial species reported to degrade glyphosate, based on the data available in Table 1 (blue bars). The red stars (*) indicate the additional lineages, for which no glyphosate-degrading species have been reported yet, and which, based on our KEGG and BLASTp searches, may have glyphosate-degrading activity. Further research is required to confirm this activity.

3.2. Protein Conservation across Prokaryotes

The entries in KEGG for the proteins of the C-P lyase complex belong to a wide range of bacterial phyla (Figure S2A,C). The bacterial species expected to express C-P lyase based on KEGG (Figure S2) belong to more bacterial groups than the bacterial species reported in the literature as degrading glyphosate (Table 1, Figure 2). This suggests that there are

more bacterial species with the potential to degrade glyphosate that have not been studied yet; it may also simply show conserved function against phosphonates, which may or may not include glyphosate. More specifically, the additional bacterial groups found after our KEGG search are actinobacteria, chloroflexi, clostridia, cyanobacteria, δ-proteobacteria, and ε-proteobacteria.

For Gox, as there were no relevant KEGG entries, we performed BLASTp and NCBI [90] keyword searches. We were able to find only 13 annotated Gox proteins. Those belong to α-proteobacteria, β-proteobacteria, and bacilli, but many of them are partial and the organism is not yet known (Table S2). The BLASTp search results included mostly FAD-dependent oxidoreductases, D-amino acid dehydrogenases, and glycine oxidases. Based on the percent identity of the BLASTp results, the sequences with the general term "FAD-dependent oxidases" showed higher sequence similarity to the annotated Gox sequences than the "D-amino acid dehydrogenases" and GOs; GOs demonstrated the lowest average percent identity (Figure S3).

The BLASTp searches for Gox without filtering per group showed fewer bacterial groups than those reported in the literature, namely, sequences only from α-, β-, and γ-proteobacteria (Figures S2B, S4 and S5). However, when we performed the searches per bacterial group, the diversity of the results increased significantly (Figure S2B,D). In addition to the aforementioned groups, we found entries belonging to verrucomicrobia, fusobacteria, aquificae, and planctomycetes. Figures S4 and S5 show distinctive changes in the phylogenetic distribution of the BLASTp results. Although there are no known motifs of Gox responsible for glyphosate degradation, these BLASTp results are worth investigating further for such a potential.

3.3. Conserved Residues in C-P Lyase

Multiple alignments of each of the proteins of the C-P lyase complex revealed a large number of conserved residues (Figure S6). The results were compared with the C-P lyase complex of *E. coli* (4X6B), which has been extensively studied previously. Although *E. coli* cannot degrade glyphosate, PhnI and PhnJ proteins show a high degree of conservation with glyphosate-degrading bacteria (*Sinorhizobium meliloti)* [75]. A detailed description of the discovered conserved residues, previous mentions in the bibliography, and the secondary structure of each region are found in Table 2. The position of the highly conserved residues on the tertiary structure of the enzyme is highlighted in Figure S7.

From our alignments, many previously unreported conserved residues were discovered, which could be significant for the function of the enzyme. Some of these residues are close to the active site of PhnJ, or are part of the domains CMD, CID (PhnJ), BBD, and NTD (PhnI). The function of certain conserved residues has been suggested previously and, by proximity, we can deduce the function of several of the newly identified conserved residues. For example, residues G208, R209, G47, G49, V125, T50, and G51 were previously found, using molecular docking, to interact with AMPA via hydrogen bonds [43]; we find conservation of these residues, which lends support to their important role, and we suggest that they also surround and may help stabilize the Fe_4S_4 cluster. Also, in addition to the Cys residues surrounding Zn, Gly245 is conserved right next to Cys244, suggesting that this may also contribute to stabilizing Zn. The loop containing the universally conserved Gly32 also contains two more conserved residues (Pro31, Gln34), which could participate in receiving the intermediate radical while catalyzing the substrate formed by SAM. The fact that the CMD domain only contains three conserved residues matches the hypothesis that it might detach from the core of PhnJ during interaction [37]. Conserved His residues in the interface of PhnJ and PhnI also coordinate a second Zn molecule. From our alignments, more residues were found to be conserved in this region in both the PhnJ and PnhI proteins, suggesting that they contribute to the overall structure around Zn. The large number of highly conserved residues supports the assumption that this could be a potential second active site of the C-P lyase complex [37]. PhnG is generally not highly conserved. However, there are a small number of conserved residues in the C-terminal part, which is reported to interact with PhnI [47]. Notably, there is a set of conserved residues, remote

from the rest of the C-P lyase core complex, which does not seem to interact with any substrate or other proteins of the complex.

Table 2. Conserved residues of the C-P lyase complex of *E. coli*, identified in alignments from this study, compared to those described in the literature, for the proteins PhnJ, PhnI, and PhnG. The blue highlighted residues are the unique ones found in this study, which are not mentioned in the literature. The green highlighted residues are those mentioned in the literature which were not highly conserved in our alignments (i.e., scores 10 or 11 based on Jalview, see methods). The asterisk (*) denotes that P36 is at the border of loop2 and a b-sheet that precedes the loop.

PhnJ			
Secondary Structure	**MUSCLE Alignment Residues Identified in Alignments from This Study**	**Conserved Residues as Given by Seweryn et al. [37]**	**Residues Predicted In Silico to Participate in Interaction with AMPA and GLP [43]. HB: H Bond HI: Hydrophobic Interaction**
Residues Surrounding the Fe_4S_4 Cluster			
loop 1	A207, G208, R209, E210	-	AMPA: G208 (HB), R209 (HB)
loop 2	P36 *, P40, P43 Near the cluster: G47, G49	-	AMPA: G47 (HB), G49 (HB)
loop 3	Q124, V125, P126	-	AMPA: V125 (HB)
helix 1	T50, G51, G52 More distant: Q54	-	AMPA: T50 (HB), G51 (HB)
Residues surrounding Zn			
loop 4	G245	C241, C244	Y250 (HI); more distant to Zn, S267 (HB)
β sheet loop		C266	
helix6		C272	
Residues surrounding universally conserved residue G32			
loop 5	P31, Q34	G32	
CID—Central insertion domain—contacts both of the central PhnI molecules			
loop 3	L131, E135		
helix 2	H145	129–169	
helix 3	Y150, L157		
CMD—C-terminal mini domain—stabilized by Zn ion and conserved C residues			
β hairpin	E253 (loop), G260 (β sheet)	236–281	
His108 coordinate Zn ion with 2 His from PhnI			
loop 6	R107, R109 More distant: P111, F112, L115	H108	GLP: H108 (HB), R109 (HI)
loop 7	P187	-	
helix 5	P189, D192	-	GLP: P189 (HI)
loop 8	D70, Q71, G72	-	GLP: Q71 (HB)
Two of the residues (PhnI His328 and His333) coordinate the zinc ion directly PhnJ His108, PhnI His328, and PhnI His333 are located in a cavity between PhnI and PhnJ			
β strand 1	K67, V68, I69, D70		
loop	Q71, G72		
β strand 2	I104, Q105, R107		
loop	R109	(H108)	
loop (Bhatt)	P174, F232		Y171 (HI)
PhnI			

Table 2. *Cont.*

Secondary structure	MUSCLE alignment Residues not reported by [37], identified in alignments from this study		Conserved residues [37]
Residues surrounding the Fe_4S_4 cluster			
loop 1 loop 2	A55	G278	-
NTD—N-terminal domain			
β strand 1	Y2, A4		
helix 2	G7, G8, A11		
helix 3	E51		
loop 1	A55		
helix 4	A60, A63, Q66		1–88
loop 4	G69		
helix 5	E73, A74, R82, T84		
β strand 2	K87		
His108 coordinate Zn ion with 1 His from PhnJ			
loop 5	P332		-
helix 6	Y334		H333
helix 7	F325, K330 Further away: D318, G324		H328
C-terminal			
helix 8	R189, R198, Y209, R213		
loop 6	G214, G216, H219		
β strand 3	G223, E224, R226, G228		188–318
β strand 4	G246, E252		
β strand 5	G272, G274		
loop 7	G278		
helix 9	E281, K283, M287		
helix 10	F312, H316		
helix 11	D318		
PhnG			
Secondary structure	**MUSCLE alignment Residues not reported by [37], identified in alignments from this study**		**Conserved residues [37]**
β-hairpin and C-terminal helix form a molecular clamp that connects to a groove in PhnI			
β strand 1	G63	-	-
β strand 2	G80	-	-
helix	A96, D99, A100	-	-
loop	V139, F141	-	-

Most residues which are reported to be conserved in the literature and play a significant role in the function of the enzyme were also conserved in our alignments. However, five residues of PhnJ that are expected to interact with glyphosate and AMPA in silico [43],

and three of the residues participating in the conserved Cys motif of PhnJ, are not absolutely conserved in our alignment. This discrepancy may be because we used more sequences in the alignments compared to Seweryn et al. [37]. Cys244 and Cys266 are present in 96% of the sequences, but they are replaced by amino acids with the same properties (Ser or Thr and Ile or Thr, respectively). The most important change is noticed for Cys272, which plays a significant role in the function of the enzyme as it is the last Cys residue of the conserved motif CX2CX21CX5C [37]. According to our results, position 310 of the multiple alignment, which corresponds to Cys272 of *E. coli*, was not absolutely conserved in all sequences and it was replaced by either Met or Leu. The sequences that did not correspond to Cys in this position were:

- cyb—*Synechococcus* sp. JA-2-3B'a(2-13) (bacteroidota)—Met269;
- muh—*Mucilaginibacter celer* HYN0043 (bacteroidota)—Leu276;
- shv—*Salinicoccus halodurans* H3B36 (bacilli)—Met269;
- crn—*Carnobacterium* sp. 17-4 (bacilli)—Met27.

This raised the question of whether the motif is present in another part of the sequences, and whether the rest of the sequences actually have the conserved motif. Although Cys was conserved in this position for the rest of the entries, in some cases the number of residues between the conserved Cys was different than that determined for the CX2CX21CX5C motif. For this reason, we searched for the motif CX2CX21CX5C in all of our sequences: it was found in only 77 out of 93 PhnJ sequences in total. The 16 sequences in which the motif was not present are shown in Table S3. However, as the typical motif for SAM enzymes is CX3CX2C, and variations of this motif (CX4CX2C, CX5CX2C, CX3CX3C, CX14CX2C) can also be observed [91], we also searched for these motifs in our sequences. The CX3CX2C SAM typical motif was not found in any of the sequences, and none of the sequences without the CX2CX21CX5C motif contain any alternative motif. Only two sequences contained the alternative motif CX5CX2C and, interestingly, both have the motif of PhnJ too: hbe—*Halomonas beimenensis* NTU-111 (γ-proteobacteria), and stea—*Sporolactobacillus terrae* DRG1 (bacilli).

3.4. Structure of Gox

There is no available experimental structure for Gox in PDB. The only attempt at a structural approach used Phyre2, based on the amino acid sequence of *Ochrobactrum* sp. G1 (ACZ58378.1) [43]. We created structural models based on AlphaFold Colab and I-TASSER, which present similarities with Phyre2 (Figure 3). According to the evaluation based on QMEANDisCo (Figure S8), the best model is produced by AlphaFold, a conclusion that is supported by the recent assessment of structure prediction models (CASP14), referring to the performance of AlphaFold [92]. Phyre2 and AlphaFold show significant differences in specific regions of the predicted structure, which end up around the surface of the structure (Figures 3 and S9). Based on the docking results of Bhatt et al. [43], glyphosate is expected to bind to the center of the protein. There are slight differences between the three models; for example, R358 is part of a loop in I-TASSER but on a beta sheet in AlphaFold and Phyre2, and M388 is in a helix in Phyre2, but part of a loop in I-TASSER and AlphaFold. As glyphosate is a small molecule and these accumulative small changes may alter the shape of the binding site, this can have an impact on the functionality of the proposed binding site on each calculated structure. It is also not known yet if Gox acts as a monomer or dimer/polymer; in the latter case, the peripheral structure may also play a role in subunit interactions.

The residues hypothesized by Bhatt et al. [43] to interact with glyphosate were not consistently conserved in our multiple alignments (Table 3). None of the residues were conserved for all datasets, and only N47 and R358 were highly conserved for the results of both BLASTp_nr_NOGROUP and BLASTp_ref_NOGROUP. Residues like V16, A43, and M388 were conserved in less than 50% of the sequences in all 4 alignments. Therefore, further structural and docking studies are needed in order to obtain a more precise model of the Gox active site.

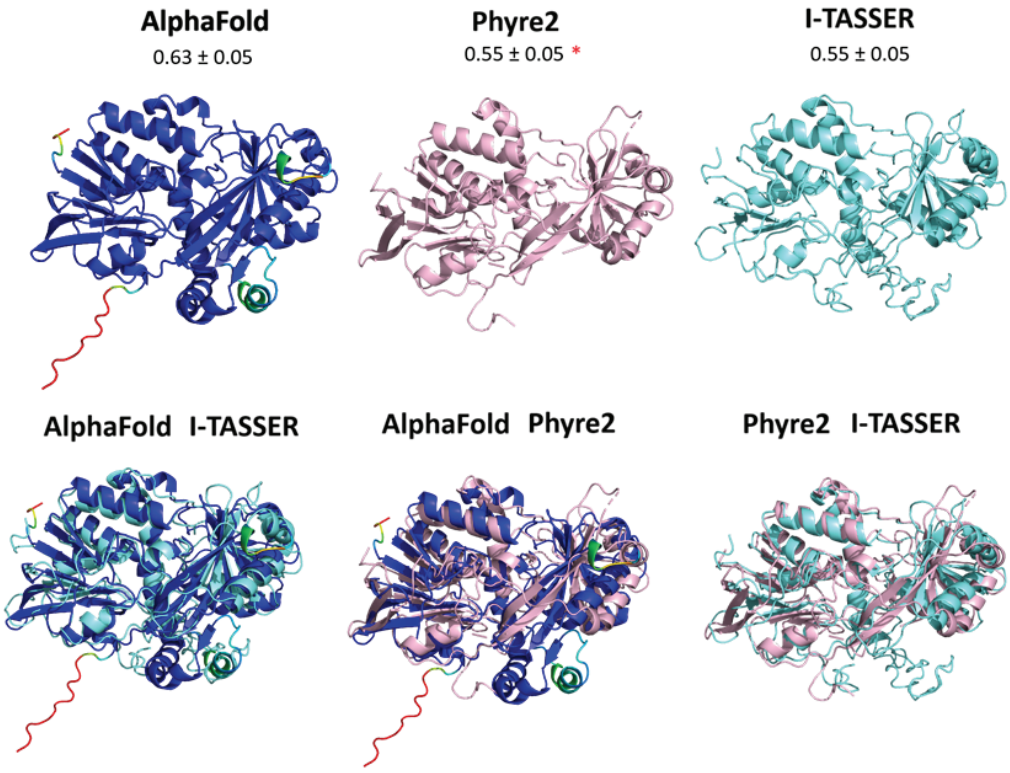

Figure 3. The computed 3D structures of Gox from *Ochrobactrum* sp. G-1 (NCBI Accession number ACZ58378.1) using AlphaFold, Phyre2, and I-TASSER. Top panel: individual models with the ranking for each program (local quality estimate, QMEANDisCo global ranking). * Although the performance of I-TASSER and Phyre2 seems to be the same, it is important to note that part of the structure is missing in Phyre2, which has a significantly low score in AlphaFold and I-TASSER (Figure S8). Bottom panel: Structures aligned using PyMOL.

Table 3. Conservation of residues hypothesized by Bhatt et al. [43] to interact with glyphosate in our multiple alignments. Green crosses show the residues of the computed binding site that are highly conserved in each of our own multiple alignments (corresponding to a Jalview score of 10 or 11).

Bhatt et al., 2021	BLASTp_nr_ NOGROUP	BLASTp_ref_ NOGROUP	BLASTp_nr_ GROUP	BLASTp_ref_ GROUP
G14		+		+
I15				
V16				
A43		+		
S44				
N47	+	+		
R358	+	+		
G384				
G387	+			+
M388				

4. Conclusions

In this study, based on BLAST and KEGG searches, additional lineages were identified, which are worth investigation for glyphosate-degrading activity, as no glyphosate-degrading species have been reported from these lineages yet. Furthermore, additional conserved residues have been identified for each protein of the C-P lyase complex. Further studies would be useful to determine their role in the structure and function of the complex. Interestingly, Cys272 of PhnJ from *E. coli*, an important residue for the function of the active site of C-P lyase, is overall conserved but not present in all PhnJ sequences, raising the question of whether those PhnJ sequences have an alternative mechanism or if they have a

different activity. Given the limited information on glyphosate degradation through the C-P lyase pathway [35], further studies of the C-P lyase pathway and the participating genes are needed, e.g., regarding the efficiency of the enzyme and the dependence of the *phn* gene expression on Pi deficiency.

Oxidoreductases are a large group of versatile enzymes [93] with conserved domains of low sequence similarity. There is a big research gap in the amino acid sequences of proteins known to degrade glyphosate, as most studies of glyphosate-degrading activity are based on detecting the degradation products with no further analysis at sequence level or identification of the responsible protein (Table 1). Bioinformatics analysis of already available biological data can contribute to the knowledge about Gox proteins. There are only a few annotated Gox proteins and we lack information about their reaction mechanism, the participating residues, and even the biological function in the bacteria which express them. The BLAST results show that Gox sequences are more similar to "FAD-dependent oxidases". An experimentally determined Gox structure is not yet available and the binding site of glyphosate to the protein is not known yet. Here, we compare models based on three programs. The majority of the residues previously proposed to interact with glyphosate are not highly conserved in our alignments. Therefore, it would be interesting to create a more detailed structure in silico, which can be used for docking, in order to study the interacting residues and to search for enzymes with similar active sites. This could lead to the discovery of enzymes with higher efficiency, being better candidates to be applied in situ for the remediation of contaminated sites.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria3040021/s1, Figure S1: Tertiary structure of the C-P lyase core complex; Figure S2: Distribution of C-P lyase and Gox across the major bacterial lineages; Figure S3: Average percent identity values between the resulting amino acid sequences from our BLASTp searches and the annotated Gox proteins; Figure S4: Phylogenetic distribution of the BLASTp results against the nr database using the Gox ACZ58378.1 as a query sequence; Figure S5: Phylogenetic distribution of the BLASTp results against the Refseq database using the Gox ACZ58378.1 as a query sequence; Figure S6: Conserved residues in C-P lyase deduced from the MUSCLE alignments in this study, shown per protein subunit; Figure S7: Conserved residues in C-P lyase marked on the 3D structure of the enzyme; Figure S8: Scoring of the computed Gox structures; Figure S9: Phyre2 and AlphaFold show significant differences in specific regions of the predicted structure; Table S1: Methodology of the choice of the amino acid sequences for Gox homologs; Table S2: The annotated Gox protein types in NCBI. Table S3. KEGG entries of the PhnJ sequences which lack the motifs CX2CX21C and CX2CX21CX5C.

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