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Emerging trends and advances in the socioeconomic applications of beneficial microbes

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Emerging trends and advances in the socioeconomic applications of beneficial microbes

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Editorial: Emerging trends and advances in the socioeconomic applications of beneficial microbes

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Editorial on the Research Topic
Emerging trends and advances in the socioeconomic applications of beneficial microbes

Plants interact with a wide range of microorganisms (bacteria, yeasts, filamentous fungi, viruses). Although plant pathogens have received the most attention because of their harmful effects on plant growth, development, and productivity, they represent only a small part of the microbial communities. In contrast, non-pathogenic microorganisms are abundant in Nature and can establish mutualistic relationships with their plant hosts. The United Nations General Assembly (UNGA) Science Summit contemplated, "understanding the world of microbes is imperative either to curb dangerous effects or to harness their power for healthier life, for sustainable energy sources, for biodiversity, for tackling climate change and for solving hunger problems", one of the key objectives of the United Nations Sustainable Development Goals (SDGs). The initiatives by UN SDGs cointegrate microbial sciences and biotechnologies for a better life and facilitate innovations, health and wellbeing, hunger elimination, clean water and sanitation, industry, and infrastructure, providing sustainable solutions. This editorial discusses the latest advances in microbial biotechnologies and state-of-the-art concepts in harnessing microbial traits for sustainable livelihood, and addressing societal concerns, defining the future trajectory of this emerging field.

The field of plant-microbe biotechnologies is rapidly evolving, and significantly contributing to agriculture, healthcare, and environmental subsistence including other biotechnological innovations. The diverse microorganisms produce different functional foods and other high-value food ingredients, including pigments, enzymes, and food flavors, and are also vital in improving crop yield and productivity in agriculture. In response to global food demand, the beneficial implications of microorganisms in agriculture need to be promoted via the use of microbial inoculants as biofertilizers (and reduce chemical fertilizer usage), soil-carbon restoration, and genetic manipulation studies to harness their full potential. In healthcare, many drugs, including anticancer drugs and antibiotics, are microbe-derived, and microbial platforms are utilized as biofactories for the production of novel drugs and proteins via recombinant DNA technologies. Recent

research has also highlighted the importance of gut microorganisms in digesting food components and vitamin production for human health. In the field of environmental management, microorganisms-assisted remediation of contaminated water has been successful in improving water quality and sanitation. Furthermore, microorganisms assist in biofuel production and act as a direct source of clean and affordable energy, produce substances and metabolites of high industrial importance, bioremediate environmental hazards and plastics, and enhance plant productivity and stress tolerance.

Decades of agricultural intensification have boosted crop yields at the expense of soil health and microbial diversity, jeopardizing global food security. To address this Research Topic, a study in West Bengal, India (Mukhopadhyay et al.), explored the potential of a novel multi-strain consortium of plant growth-promoting (PGP) *Bacillus* spp. for soil bioaugmentation. In this work, a composite inoculum of *Bacillus zhangzhouensis* MMAM, *Bacillus cereus* MMAM3, and *Bacillus subtilis* MMAM2 was introduced into an over-exploited agricultural soil and implications on the improvement of vegetative growth and yield-related traits of *Glycine max* (L) Meril. plants were evaluated, growing them as model plant, in pot trial condition. The study's findings demonstrated significant improvements in plant growth and soil microbial diversity when using the bacterial consortium in conjunction with vermicompost. Metagenomic analyses revealed increased abundance of many functional genera and metabolic pathways in consortium-inoculated soil, indicating enhanced soil biological health.

In a second study Debnath et al. aimed to understand plant-bacteria interactions that enhance plant resistance to environmental stressors, with a focus on maize (*Zea mays* L.) and its vulnerability to various pathogenic organisms. The potential of 1-amino-cyclopropane-1-carboxylic acid (ACCA) as a compound to boost maize's resilience against stressors and pathogens is hypothesized through an empirical computational study and needs to be confirmed by biological studies conducted for example in greenhouses.

New microbial strains interacting with plants are isolated every day. The third article (Patakova et al.) of this Research Topic presents new information about the genome and phenotypic characteristics of *Pantoea agglomerans* strain DBM 3797, isolated from fresh Czech hop (*Humulus lupulus*). *P. agglomerans* DBM 3797 was cultured under aerobic and anaerobic conditions, its metabolites were analyzed by HPLC and it was tested for plant growth promotion abilities, such as phosphate solubilization, siderophore, and indol-3-acetic acid productions. In addition, genomic DNA was extracted, sequenced, and *de novo* assembly was performed. Further, genome annotation, pan-genome analysis, and selected genome analyses, such as CRISPR arrays detection, antibiotic resistance, and secondary metabolite genes identification were carried out. As concluded by authors, this strain has a number of properties potentially beneficial to the hop plant, however, its safety profile needs to be addressed in follow-up research.

In another article by Dhar et al., focus is put on *Rhododendron ferrugineum* L., Nepal's national flower and Uttarakhand's state tree, thriving in high-altitude mountain ecosystems. Leaf anomalies were traced back to the pathogenic fungus *Curvularia tuberculata*, marking the first documented case of its impact on *R. ferrugineum* in India. Overall, this study calls for proactive measures to protect *R. ferrugineum's* cultural and ecological heritage and emphasizes the significance of interdisciplinary approaches (including researches to identify a biological control agent able to manage the pathogenic fungus *Curvularia tuberculata*) in addressing emerging ecological threats.

A second computational study is presented in Perveen et al. with investigations on the synergistic action of plant natural products curcumin and mangiferin against *Bacillus anthracis*. Mangiferin is a natural C-glucosylxantone compound that has many substantial curative potentials against numerous illnesses including cancers. Similarly, the anti-cancer effects of curcumin and its analogs have caught many enthusiasts over the last two decades. Screening antibacterial properties of these two compounds, employing high-throughput screening, authors identified potential binding sites on *B. anthracis*. Molecular docking revealed that curcumin and mangiferin, when synergistically combined, exhibited strong binding affinities at different sites on the bacterium.

The intricate relationship between cancer and bacteria has garnered increasing attention in recent years. For example, the gut microbiome is implicated in the pathogenesis of colorectal cancer (CRC), but the full scope of this dialogue is still unknown in 2024. While traditional cancer research has primarily focused on tumor cells and genetic mutations, emerging evidence highlights the significant role of microbial communities within the tumor microenvironment in cancer development and progression. The review Lu and Tong aims to provide a comprehensive overview of the current understanding of the complex interplay between cancer and bacteria. By conducting a thorough analysis of the existing literature, Lu and Tong underscore the multifaceted and intricate relationship between bacteria and cancer. Understanding this complex interplay could pave the way for novel therapeutic approaches and preventive strategies in cancer treatment.

The gut microbiota, intensely intertwined with mammalian physiology, significantly impacts health, productivity, and reproductive functions. The normal microbiota interacts with the host through the following key mechanisms: acting as a protective barrier against pathogens, maintain mucosal barrier integrity, assisting in nutrient metabolism, and modulation of the immune response (Khan et al.). This review emphasizes the critical ecological roles of mammalian microbiota, highlighting their essential contributions to health, productivity, and reproductive success. By integrating human and veterinary perspectives, it demonstrates how microbial communities enhance immune function, metabolic processes, and hormonal regulation across species, offering insights that benefit both clinical and agricultural advancements

The following experimental article is also about microbiota structure, with a special focus on reproductive tract of cattle. The top three bacterial phyla in bovine reproductive tract were *Proteobacteria*, *Firmicutes,* and *Bacteroidetes*, accounting for more than 85%. From the vagina to the uterus, the relative abundance of *Proteobacteria* gradually decreased, while

that of *Firmicutes* gradually increased (Teng et al.). These findings lay a foundation for a comprehensive understanding of the structure of the genital tract microbiota of cows and its regulatory mechanisms.

The interplay between gut microbiota and host health is crucial for maintaining the overall health of the body and brain. Lot of microorganisms are involved, such as *Akkermansia muciniphila* which seems a promising next-generation probiotic with clinical application prospects. Emerging studies have reported various beneficial effects of *A. muciniphila* including anti-cancer, delaying aging, reducing inflammation, improving immune function, regulating nervous system function, whereas knowledge on its roles and mechanism in infectious disease is currently unclear. In summary, Li et al. believe that *A. muciniphila* is a promising therapeutic probiotic that may be applied for the treatment of a variety of infectious diseases.

Beneficial microbes may also have socioeconomic applications through industrial biotechnology, with the production of various metabolites, or enzymes. Chitin and chitooligosaccharides have been widely applied in food-related fields, with biodegradable, biocompatible, nontoxic, antimicrobial, and antioxidant activities. Processing and biorefinery should still being improved and the article from Xie et al. refine the taxonomic description of *Rhodococcus indonesiensis* and investigates its application in converting chitin into chitosan. The chitin deacetylase (*Ri*CDA) activity of the strain T22.7.1^{T} was optimized, and the enzyme was isolated and purified from the fermentation products. Product analysis revealed that *Ri*CDA treatment increased the deacetylation degree (DD) of natural chitin to 83%, surpassing that of commercial chitosan.

The Research Topic has been an advocate in providing key insights and bridging the knowledge gaps in understanding the dynamics of plant-microbe interactions. It encouraged the submission of high-quality research articles and reviews covering the most recent advances in microbiology. We are pleased to note that our Research Topic has attracted contributions from many highly regarded researchers deeply involved for many years in Microbiology around the world, including from Bangladesh, China, Czechia, Egypt, France, Germany, India, Malaysia, Romania, Saudi Arabia, and the USA. We received 16 submissions, 10 of which were accepted (seven original research articles, three reviews) for publication after rigorous peer-review, with a total of 93 authors.

Author contributions

LD: Conceptualization, Validation, Writing – original draft, Writing – review & editing. PT: Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exploring the efficacy of 1-amino-cyclopropane-1-carboxylic acid (ACCA) as a natural compound in strengthening maize resistance against biotic and abiotic stressors: an empirical computational study

Sandip Debnath[1]*, Abdallah M. Elgorban[2], Ali H. Bahkali[2], Rajalakshmanan Eswaramoorthy[3], Meenakshi Verma[4], Pragya Tiwari[5], Shifa Wang[6], Ling Shing Wong[7] and Asad Syed[2]*

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Objective: This study aims to understand plant-bacteria interactions that enhance plant resistance to environmental stressors, with a focus on maize (*Zea mays* L.) and its vulnerability to various pathogenic organisms. We examine the potential of 1-amino-cyclopropane-1-carboxylic acid (ACCA) as a compound to boost maize's resilience against stressors and pathogens.

Background: With the growing global population and increased food demand, the study of endophytes, comprising bacteria and fungi, becomes crucial. They reside within plant tissues, affecting their hosts either beneficially or detrimentally. Agrobacteria are of specific interest due to their potential to contribute to developing strategies for plant resistance enhancement.

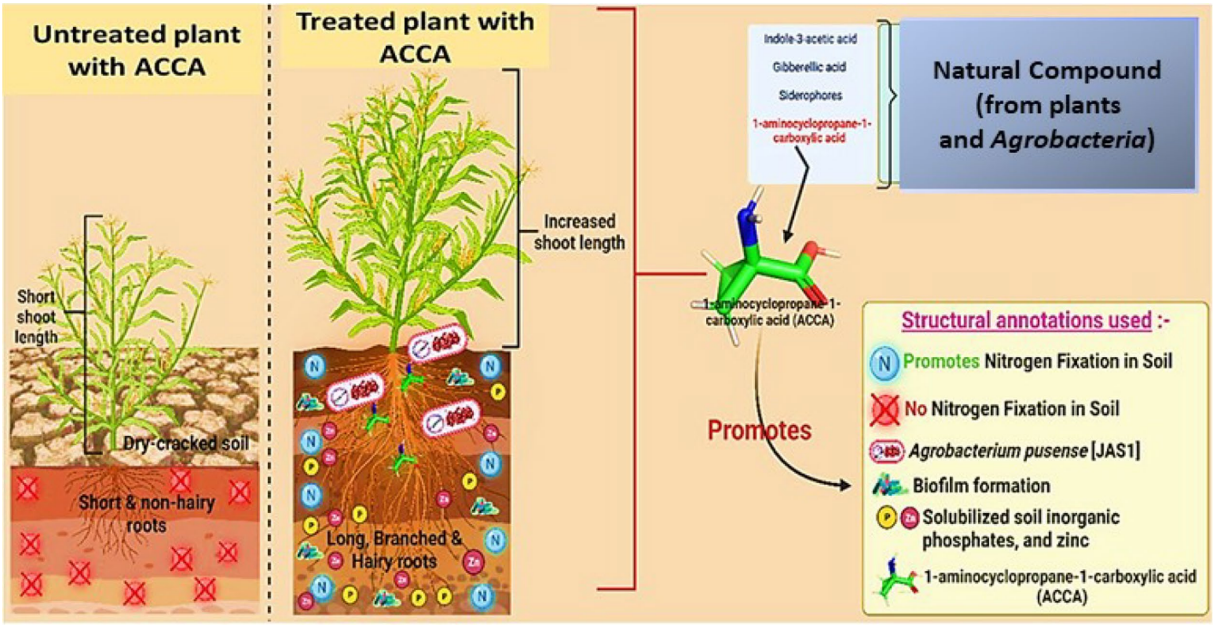
Methods: We conducted exhaustive research on the defense-related proteins and mechanisms involved in maize-pathogen interactions. The efficacy of ACCA as a natural-compound that could enhance maize's resistance was examined.

Results: Our research indicates that ACCA, having a binding energy of −9.98 kcal/mol, successfully strengthens maize resistance against pathogenic assaults and drought stress. It plays a crucial protective role in maize plants as they mature, outperforming other ligands in its effectiveness to improve productivity and increase yield.

Conclusion: Applying ACCA to maize plants has considerable potential in enhancing their resilience and tolerance to stress, proving to be an effective strategy to boost crop yield and productivity. This could help address the increasing global food demand. However, more research is needed to optimize ACCA application methods and to gain a comprehensive understanding of its long-term effects on maize cultivations and the environment.

KEYWORDS

1-amino-cyclopropane-1-carboxylic acid, drought resistance, fungal attack, microbial attack, molecular docking, molecular dynamics, pathogenic attack



GRAPHICAL ABSTRACT

Introduction

Maize (*Zea mays* L.) is an important crop cultivated year-round in India, particularly during the Kharif season, where it accounts for 85% of the country's cultivation (Warham et al., 1996; APEDA, 2023). Despite being classified as a grain, corn kernels are commonly used as a vegetable or starch. Maize thrives in various climates across India, including semiarid, sub-humid, and humid regions. It is particularly popular in the low- and mid-hill sections of the western and northeastern regions. Traditional maize-growing areas in India include Uttar Pradesh, Bihar, Madhya Pradesh, and Rajasthan, while non-traditional areas include Karnataka and Andhra Pradesh (Ranjith et al., 2022). Maize production in India faces significant challenges due to pathogen infections, which pose a threat to global food supplies, especially in maize-producing regions. These pathogens cause severe ear and leaf diseases in corn plants, leading to a decline in crop production and quality (The CIMMYT Maize Program, 2004; Pechanova and Pechan, 2015). Additionally, fungal infections and mycotoxin contamination could cause detrimental effects on human and animal health through direct infection or the consumption of contaminated food and feed (McGee, 1988). One of the prevalent diseases in India is anthracnose, which causes leaf blight and stem-rot in maize plants, resulting in substantial agricultural losses and various diseases (Warham et al., 1996). Another significant threat to maize cultivations is the presence of *Spodoptera frugiperda*, a maize pest that causes annual losses of 8.3–20.6 million tons. This destructive beetle is native to tropical and subtropical regions of the United States (Wang et al., 2020; Molina-Romero et al., 2021; Debnath et al., 2022a,b; APEDA, 2023; National Center for Biotechnology Information, 2023). To combat these challenges, researchers have explored the potential of utilizing beneficial compounds derived from *Agrobacterium pusense* strain JAS1. This strain is known for its nitrogen fixation and biofilm formation capabilities (Kaur et al., 2022). The strain produces various chemicals contributing to plant immune-boosting, root hair growth, and shoot development. One such compound of interest is 1-amino-cyclopropane-1-carboxylic acid (ACCA). ACCA is an ethylene precursor in plants and plays a crucial role in various physiological processes such as fruit ripening, senescence, and stress response (National Center for Biotechnology Information, 2023). Ethylene, a gaseous plant hormone, regulates developmental processes and serves as a signaling molecule in response to biotic and abiotic stresses.

In the study, virtual screening was conducted to identify potent compounds, including plant-derived and Agrobacteria-derived compounds. The focus was primarily on ACCA due to its biochemical similarity to cyclopropane carboxylic acid (National Center for Biotechnology Information, 2023). ACCA was the most effective compound against maize, as it induced a virulence system that enhanced the plant's defense against pathogenic attacks. By modulating ethylene biosynthesis, ACCA can alter plant defense mechanisms, allowing plants to cope better with various stress factors (Jorgensen et al., 1996; Bowers et al., 2006; Chow et al., 2008; Shaw et al., 2010; Shivakumar et al., 2010; Meng et al., 2011; Ferreira et al., 2015; Kaur et al., 2022; Debnath et al., 2022a,b; Mukerjee et al., 2022b,c; APEDA, 2023; Debnath et al., 2023).

Considering the wide range of microbial pathogens causing damage to maize cultivations, this article proposes a strategy to enhance maize's virulence and stress tolerance by spraying plants with ACCA, as depicted in Figure 1. This research's overarching aim is to promote sustainable agriculture by minimizing crop losses through the application of a natural chemical compound, 1-amino-cyclopropane-1-carboxylic acid (ACCA), which has a minimal environmental footprint. This approach marks a significant stride in the development of environmentally conscious agricultural practices. However, it is crucial to conduct additional investigations to optimize the application methodologies of ACCA and thoroughly evaluate its long-term implications on maize cultivation and the broader ecosystem.

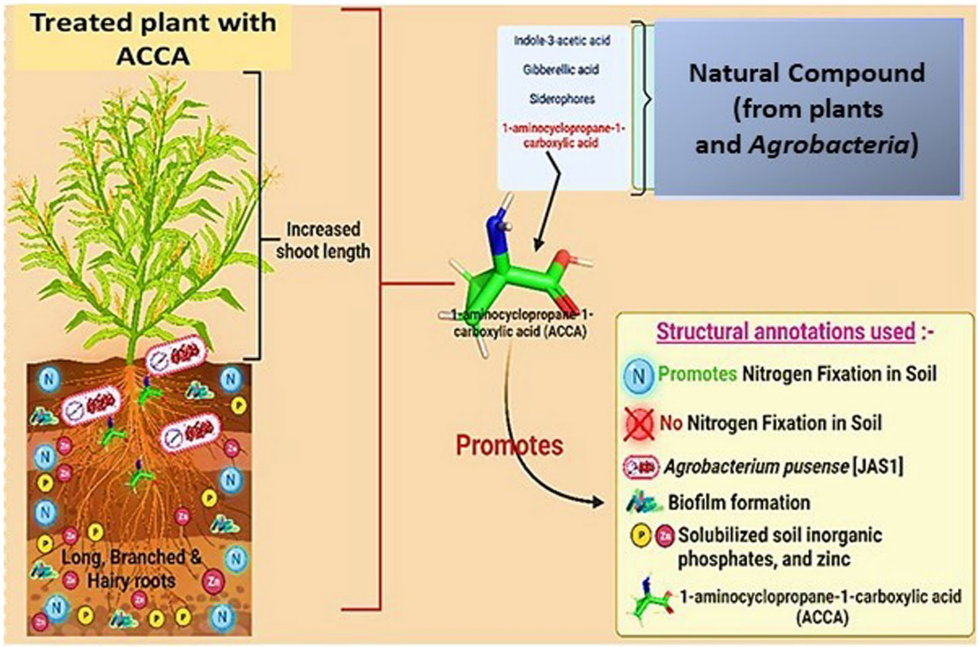


FIGURE 1
Biotechnological approach which promotes *Zea mays* to attack natural chemical [1-*amino*-cyclopropane-1-carboxylic acid (ACCA)] induces beneficial role in the plant (created with Adobe Illustrator and BioRender.com).

To elucidate the finer molecular interactions between ACCA and maize plants, we propose the utilization of molecular dynamics (MD) simulations. MD simulations are a class of computational strategies used to model the temporal behavior of atoms and molecules. These simulations can provide deep insights into the structural and functional properties of chemical compounds.

The Kharif season, prevalent in the Indian subcontinent, typically begins with the onset of the monsoon in the months of June and July, and extends until October and November. Crops sown during this period are often rain-dependent, and their growth coincides with the heavy monsoon rains. Some of the primary crops grown during this season include rice, maize, sorghum, and various types of pulses. Agriculture plays a pivotal role in the Indian economy, accounting for a significant proportion of the country's gross domestic product (GDP) and providing employment to a vast majority of the population. India's diverse agro-climatic zones allow for the cultivation of a wide variety of crops throughout the year.

In terms of agricultural production and productivity, India ranks among the top global producers for several key crops. The country is the largest producer of pulses, the second-largest producer of rice, wheat, and several fruits and vegetables, and holds a significant position in the production of spices and plantation crops such as tea and coffee.

While the country's agricultural sector has achieved significant strides, it also faces numerous challenges. These include issues related to climate change, water scarcity, soil degradation, and the need for increased yield to meet the demands of a growing population. The adoption of sustainable and resilient farming practices, such as the use of bioactive agents like 1-amino-cyclopropane-1-carboxylic acid (ACCA) for crop protection, can play a crucial role in addressing these challenges and promoting the sustainable growth of the sector.

In the context of our study, MD simulations are invaluable for exploring the binding affinity and stability of the ACCA complex with crucial maize proteins and receptors. These simulations can capture the molecular interactions of ACCA with its target, offering insights into the molecular mechanisms that contribute to ACCA's efficacy in enhancing the resistance and stress tolerance of maize plants. This understanding of the molecular underpinnings of their interaction can pave the way for improved crop protection strategies and lead to advancements in sustainable agriculture practices (Martyna et al., 1992, 1994; Toukmaji and Board, 1996; Kagami et al., 2020; Umar et al., 2022; Mukerjee et al., 2022a).

Moreover, the study also considered other compounds with potential applications in enhancing maize's resistance to pathogens and stress. These compounds include gibberellic acid, indole-3-acetic acid, citric acid (as a siderophore), hydroxamate, dextran, and xanthan. Gibberellic acid is a plant hormone known for its role in promoting plant growth, while indole-3-acetic acid is involved in various plant processes, including cell division and elongation. Citric acid acts as a siderophore, aiding in iron uptake by plants. Hydroxamate, dextran, and xanthan are all polysaccharides that have been shown to have beneficial effects on plant growth and stress tolerance. These compounds can be further investigated through experimental studies and molecular modeling techniques to assess their potential to enhance maize's resistance to pathogens and stress. By expanding the scope of the research to include these compounds, a comprehensive understanding of their individual and combined effects on maize cultivations can be obtained. Plant growth-promoting rhizobacteria (PGPR) are a class of beneficial bacteria that inhabit the rhizosphere, the region of soil directly influenced by root secretions and associated soil microorganisms. They play a crucial role in plant health and growth by a variety of mechanisms such as nitrogen

fixation, phosphate solubilization, iron sequestration, and the production of phytohormones. PGPR also enhance plant resilience against abiotic stresses like drought, salinity, and heavy metal toxicity, and biotic stresses like pathogenic infections.

The beneficial metabolites produced by PGPR include indole-3-acetic acid (IAA), gibberellins, cytokinins, and ethylene, among others. These metabolites can stimulate root growth, promote nutrient uptake, and enhance plant development. In addition to these phytohormones, PGPR can produce siderophores, which are iron-chelating molecules that aid in nutrient acquisition by sequestering iron from the soil environment, making it available for plant uptake. They also produce antibiotics and lytic enzymes, which can suppress soil-borne pathogens, further benefiting the plant.

1-Aminocyclopropane-1-carboxylic acid (ACCA) is a precursor molecule in the biosynthesis of ethylene, a plant hormone that modulates plant growth and stress responses. Certain types of PGPR, such as Pseudomonas and Rhizobium species, have been found to produce ACCA, which can influence the ethylene levels in plants and thereby modulate plant growth and stress tolerance.

The specific concentration of ACCA produced by these bacteria can vary widely, depending on numerous factors including the bacterial species, environmental conditions, and the stage of bacterial growth. Therefore, it's important to conduct further research to determine the optimal concentration and conditions for ACCA production by PGPR for agricultural applications. Experimental validation through *in vitro* and *in vivo* studies will be critical in elucidating these details and translating this knowledge into practical, effective strategies for enhancing plant growth and stress resilience.

In summary, the cultivation of maize in India faces significant challenges due to pathogen infections and pests. To address these issues, researchers have explored the use of plant-derived and Agrobacteria-derived compounds, such as ACCA, to enhance maize resistance and stress tolerance. Molecular dynamics simulation can be employed to investigate the molecular interactions between ACCA and maize proteins, providing insights into the mechanisms underlying their effectiveness (Sagar et al., 1847; Gopalakrishnan et al., 2018; Soni et al., 2018). Additionally, other compounds like gibberellic acid, indole-3-acetic acid, citric acid, hydroxamate, dextran, and xanthan can be investigated for their potential applications in maize cultivation improvement. Further research is needed to optimize application methods, evaluate long-term effects, and explore the combined effects of these compounds, ultimately contributing to sustainable agriculture practices and reducing crop losses.

Pathway and the regulations involved in the pathogen and stress resistance

The stress response and pathogen resistance in plants are complex, intertwined processes, which are regulated by an elaborate network of biochemical pathways. When plants encounter pathogen attack or abiotic stressors such as drought, they trigger a cascade of signaling pathways leading to the activation of defense responses. Central to these processes are plant hormones, including abscisic acid (ABA) for drought response, and salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) for pathogen resistance.

ACCA, also known as 1-aminocyclopropane-1-carboxylate, plays a crucial role in the production of ethylene, a key hormone involved in the plant's stress response. Ethylene regulates various stress responses in plants, including defense against pathogens and abiotic stress tolerance. ACCA is converted to ethylene through the action of ACC oxidase, a process that can be significantly upregulated under stressful conditions.

The function of ethylene in pathogen resistance is nuanced; it plays a crucial role in triggering defense responses against necrotrophic pathogens, but its crosstalk with SA and JA can modulate defense against other pathogen types. In stress response, ethylene collaborates with ABA, with both hormones coordinating to manage plant responses to abiotic stressors like drought.

Meanwhile, the role of ACCA in stress resistance goes beyond its function as an ethylene precursor. Recent studies have suggested that ACCA can act as a signaling molecule itself, potentially interacting with other plant defense hormones and impacting plant stress response at multiple levels. This places ACCA at a unique position within the intricate web of plant defense and stress response pathways.

However, while our current understanding of ACCA's roles in these processes is growing, it still remains incomplete. Future studies should aim to elucidate ACCA's precise interactions within the plant's hormonal network, and how these interactions influence the plant's capacity to withstand abiotic and biotic stresses. Unraveling this could pave the way for innovative strategies to enhance crop resilience and productivity.

Materials and methodology

Target protein and ligand preparation

In a recent study we found regioselective silibinin glucosyltransferase from *Zea mays*, known as UGT706F8. This enzyme, a member of the Family 1 glycosyltransferases, was found to play a crucial role in maintaining drought and stress resistance in the plant. The research identified that UGT706F8 efficiently and selectively catalyzes the synthesis of silibinin 7-O-β-d-glucoside, bypassing the traditionally complicated chemical procedures requiring multistep syntheses and protective group manipulations. The utilization of UGT706F8 enables an eco-friendly and regioselective bond formation with fully deprotected substrates in a singular reaction, greatly enhancing atom economy and sustainability. Among 18 glycosyltransferases tested for activity on silibinin, UGT706F8 was the only one displaying regioselective behavior. The enzyme was observed to function optimally at a temperature of 34°C and a pH range of 7–8. Further insights from our study, including the crystal structure of UGT706F8 and the molecular determinants of regioselective silibinin glucosylation, suggest that this enzyme holds tremendous potential for the biocatalytic production of silibinin 7-O-β-d-glucoside. This paves the way for a sustainable, large-scale production of this important pharmaceutical, while simultaneously highlighting the critical role of UGT706F8 in fortifying plant resilience against environmental stresses (Bidart et al., 2022).

Therefore, the putative target protein, which is the protein of interest in this study, was obtained from the RCSB Protein Data Bank (PDB) with the identifier 7Q3S,[1] as illustrated in Figure 2. To explore

1 https://www.rcsb.org/7Q3S

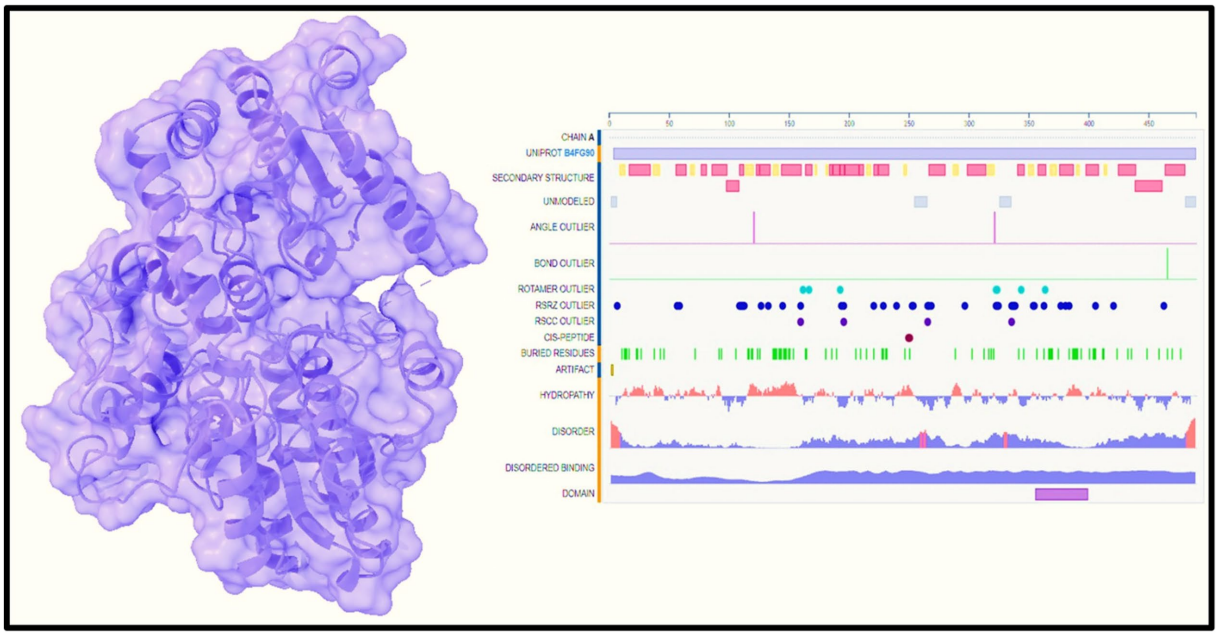


FIGURE 2
Target protein view with its sequence.

the potential binding between 1-amino-cyclopropane-1-carboxylic acid (ACCA) (Chem I. D: 65036) and the target protein 7Q3S, three-dimensional (3D) structures in the form of .sdf files were acquired from PubChem for both the protein and ACCA. The Chimera UCSF team utilized a 900-step conjugate gradient energy minimization approach to optimize the molecular structures, followed by a 1,000-step steepest-descent technique. These optimization methods are commonly employed to refine and stabilize molecular structures. The resulting molecules were then converted into .pdb format using Open Babel, a widely used tool for molecular file conversion. An additional thousand iterations of the steepest descent algorithm were applied to further minimize the energy of the molecules. To set the partial charges for the molecules, Gasteiger charges were introduced. Partial charges represent the distribution of electric charge within a molecule. Finally, the AMBER ffSB14 force field, a widely used force field optimized for protein systems in molecular dynamics simulations, was applied to the molecular models. The force field incorporates mathematical functions and parameters that describe the interactions between atoms and molecules. By employing these computational methodologies, our study aimed to gain insights into the potential interactions between ACCA and the target protein 7Q3S. These interactions can provide valuable information about the molecular mechanisms through which ACCA enhances maize resistance and stress tolerance. Understanding these mechanisms is essential for further research and the potential application of ACCA in agriculture. By studying the 3D structure of the target protein and performing molecular dynamics simulations, researchers can explore how ACCA binds to the protein and how this interaction affects the protein's function. Molecular dynamics simulations involve modeling the movement and behavior of atoms and molecules over time, allowing researchers to observe the dynamic behavior and structural changes of the protein–ligand complex. This approach can provide insights into the stability of the complex, the binding affinity between ACCA and the protein, and the specific amino acid residues involved in the interaction. Through these computational techniques, researchers can gain a deeper understanding of the molecular basis of ACCA's effects on maize plants, including its ability to enhance virulence and stress tolerance. This knowledge can guide future experimental studies and help optimize the application of ACCA in agriculture, potentially leading to improved crop productivity and sustainability.

Virtual screening of seven compounds (both from plant and Agrobacteria)

The active site of an enzyme is responsible for forming a stable bond with a specific substrate molecule (Kaur et al., 2022). In our study, we aimed to achieve a high binding affinity between our selected compound and the active site of the protein. To accomplish this, we utilized BIOVIA Discovery Studio Visualizer version 2022 (Ferreira et al., 2015), a software tool that enables visualization and analysis of molecular structures. To identify the binding location of the protein complex and generate a receptor grid, we employed AutoDockVina, a widely used docking software. AutoDockVina 4.2.6 was specifically utilized to virtually screen seven exo-polysaccharides. This screening process involved analyzing the potential binding interactions between the compounds and the macromolecule with the PDB ID: 7Q3S. The compounds were evaluated based on their binding energy scores, with the compound exhibiting the highest binding energy selected for further analysis. For each ligand, the best binding energy docked pose was chosen for re-docking and subsequent analysis. The binding energy (ΔG_bind) between the ligand and the receptor complex can be calculated using the equation:

$$\Delta G\_bind = \Delta G\_complex - (\Delta G\_receptor + \Delta G\_ligand)$$

The binding energy (ΔG_bind) represents the free energy change associated with the formation of the ligand-receptor complex. It is calculated by considering the free energy of the complex (ΔG_complex) and the free energies of the unbound receptor (ΔG_receptor) and ligand (ΔG_ligand). The binding energy provides a quantitative measure of the strength of the interaction between the ligand and the receptor. By analyzing the binding energy scores and employing this calculation, we can assess the strength of the interaction between our selected compound and the protein's active site. This information is crucial for understanding the potential efficacy of the compound in modulating the protein's function and its ability to enhance maize's virulence and stress tolerance. Through these computational approaches, we gain valuable insights into the molecular interactions between the selected compound and the protein, providing a basis for further analysis and experimental validation. The calculated binding energies can guide the design and optimization of compounds with improved binding affinity and efficacy, ultimately contributing to the development of novel strategies for enhancing maize cultivation productivity and resilience.

Molecular re-docking studies

Following the virtual screening process, the most potent natural chemical, 1-amino-cyclopropane-1-carboxylic acid (ACCA), was selected for further analysis. To construct the receptor grid, AutoDock MGL version 1.5.6 was utilized. The receptor and ligand molecules were saved in .pdbqt format, allowing future use. Vina Wizard was employed through the command line, using a grid point spacing of 2.14 Å and an exhaustiveness value of 8. The output files in .pdbqt format were examined using PyMol and Discovery Studio Visualizer 2021, allowing for the validation and improvement of the binding of the co-crystallized ligand. The target protein molecules were crucial in mediating the binding of 1-amino-cyclopropane-1-carboxylic acid (ACCA). The main objective of this study was to determine the inhibitory concentration (IC50) for each candidate molecule and utilize the results of the virtual screening to identify the contender with the strongest interaction with the target protein 7Q3S (Al Mashud et al., 2023). The IC_{50} value, which represents the concentration at which a compound exhibits 50% inhibition, can be calculated using the Cheng–Prusoff equation:

$$IC_{50} = K\_i^{*}\left(1+[L]/K\_d\right)$$

Here, K_i represents the inhibitor constant, $[L]$ is the concentration of the ligand, and K_d is the dissociation constant.

To prepare the 7Q3S structure for the docking investigation, a simplification step was performed using the steepest descent method with 1,000 steps. Subsequently, the AMBER ff4 force field was applied. This step was necessary to optimize the protein structure before initiating the docking experiments with the appropriate ligands. The protonation states of 7Q3S were neutralized prior to the experiment. All necessary preparations were completed before the docking investigations commenced. For the molecular docking experiments, AutoDock 4.2.6 was employed. Receptors and ligands were prepared, considering polar hydrogen bonds, Kollman and Gasteiger charges, and electrostatic forces. After merging nonpolar hydrogens, the receptor and ligand molecules were saved in .pdbqt format. A grid box with dimensions $X = 30$, $Y = 29$, and $Z = 32$, and a spacing of 2.14 Å, was generated. The protein–ligand complexes were docked using the Lamarckian genetic algorithm to obtain the lowest binding free energy (ΔG). By conducting these molecular docking experiments, researchers aimed to identify the most favorable binding interactions between the ligands, particularly ACCA, and the target protein 7Q3S. The lowest binding free energy values provide insights into the strength of the ligand–protein interactions, aiding in the selection of the most promising compounds for further analysis and potential applications. Overall, this comprehensive computational approach allowed for the exploration of ligand–protein interactions, helping to elucidate the binding mechanisms and potential inhibitory effects of the selected compounds on the target protein. The obtained results contribute to a better understanding of the molecular basis of ACCA's effects on maize resistance and stress tolerance, paving the way for future experimental studies and the development of novel agricultural strategies.

Molecular dynamic simulations

Schrodinger, LLC's Desmond 2020.1 was used to carry out 100 ns MD simulations of the main protein, 7Q3S, in conjunction with the ligand ACCA. In this particular system, the explicit solvent model with SPC water molecules and the OPLS-2005 force field was utilized (Bidart et al., 2022; Elgorban et al., 2023). In order to get rid of the charge, several Na^{+} ions were administered. It was decided to add 0.15 M NaCl solutions to the system to simulate the physiological environment. The NPT ensemble was generated in each simulation by applying the Nose-Hoover chain coupling method (Martyna et al., 1994). The simulations were run with the following parameters: a temperature of 300 K; a relaxation period of 1.0 ps; a pressure of 1 bar, and a time step of 2 ps after that. The Martyna–Tuckerman–Klein chain coupling system (Morris et al., 1996) barostat approach was utilized, and the relaxation duration was set at 2 ps. This technique was used to control the pressure. To predict long-range electrostatic interactions, the Colombian interaction radius was fixed at 9, and the particle mesh Ewald technique was utilized (Morris et al., 1996). The RESPA integrator was utilized to ascertain the forces that were not bonded. The root mean square deviation was used to evaluate the MD simulations' capacity to maintain stability (RMSD).

Results

Virtual screening for the most potent compound from bacteria

The ligand with the lowest binding energy score indicates the highest affinity for the target protein. In the case of our study, the ligand with the most favorable binding affinity for the protein 7Q3S was 1-amino-cyclopropane-1-carboxylic acid (ACCA), with a binding energy score of −9.98 kcal/mol. Further refinement was performed on this ligand within the binding cavity of 7Q3S. Among the seven different natural-chemical ligands tested in the screening process for the receptor protein 7Q3S, 1-amino-cyclopropane-1-carboxylic acid (ACCA) exhibited the highest potential affinity, as indicated in Table 1. This table provides a comprehensive overview of the most

TABLE 1 A list of 7 compounds (both from plant and Agrobacteria) with their docking score in kcal/moles.

Compounds (both from plant and Agrobacteria)	Gibbs free energy (ΔG) (in kcal/mol)
Gibberellic acid	−5.48
Indole-3-acetic acid	−7.54
Citric acid (as siderophore)	−4.57
Hydroxamate	−7.54
1-amino-cyclopropane-1-carboxylic acid (ACCA)	−9.98
Dextran	−5.27
Xanthan	−4.67

effective natural chemical, highlighting its potential affinity for the investigated protein. To gain a deeper understanding of the results, the interactions between the target protein and ACCA were thoroughly studied. In the protein–ligand complex, hydrogen bonding, hydrophobic interactions, and electrostatic interactions play critical roles in stabilizing the binding. Specific residues involved in these interactions were identified, and their contributions to the overall binding affinity were evaluated. This analysis helps elucidate the molecular mechanisms underlying the favorable interaction between the protein and ACCA. Furthermore, the binding mode of ACCA within the active site of 7Q3S was carefully examined. This investigation provides insights into how ACCA interacts with the protein, shedding light on the specific binding orientations, amino acid residues involved, and the nature of the interactions. Such information is crucial in understanding the structural basis of the ligand–protein interaction and can potentially guide the design of improved ligands with even higher binding affinities. A comparison of the binding modes and interactions of the other natural-chemical ligands tested in this study could yield valuable information regarding the structure-activity relationship (SAR). Understanding how different ligands interact with the target protein can assist in identifying key structural features and functional groups that contribute to their binding affinities. This knowledge can be utilized to guide future research endeavors aimed at developing more effective inhibitors or modulators for the target protein. Overall, the detailed analysis of the binding modes, interactions, and SAR provides crucial insights into the ligand–protein interactions and guides the rational design of potential ligands with enhanced binding affinities. Such information is invaluable in developing novel therapeutic agents and agricultural strategies that effectively modulate the target protein's function.

Molecular re-docking

Molecular docking is a computational technique used to determine the optimal arrangement between a macromolecule and a small molecule, aiming to identify favorable intermolecular interactions. In our study, molecular docking was employed to explore the binding interactions between the target protein and a set of exo-polysaccharides. The docking simulations were facilitated using the AutoDockVina wizard and PyRx tools, allowing us to evaluate the binding affinity of seven compounds derived from both plants and Agrobacteria, each possessing three-dimensional structures. The binding affinities of these compounds are summarized in Table 1.

Among the compounds tested, 1-amino-cyclopropane-1-carboxylic acid (ACCA) and the target protein 7Q3S demonstrated a distinct binding pocket during the re-docking experiments. The ligand, ACCA, was found to bind to the core pocket of 7Q3S with a favorable binding free energy of −9.98 kcal/mol, as depicted in Figure 3. The ligand interacts with the following amino acid residues with higher efficacy: Val17, Trp355 with pi interactions; Glu381, Serr378, Asn377, Gly292, Gln358, His378, Phe370, Ser293, Val 359 with van der Waals interactions. This result highlights that ACCA exhibits the highest binding affinity, indicating a strong interaction with the target protein 7Q3S. This information provides valuable insights into the potential effectiveness of ACCA as a promising candidate for further research and development. Moreover, conducting a comparative analysis of the binding modes and interactions of the other natural-chemical ligands tested in this study can yield crucial information regarding their structure-activity relationship (SAR). By examining how different ligands interact with the target protein, we can identify key structural features and functional groups that contribute to their binding affinities. This knowledge can serve as a guide for future research endeavors, aiding in the design and development of more effective inhibitors specifically tailored for the target protein. In summary, molecular docking served as a valuable tool in our study, enabling the identification of the optimal binding configuration between the target protein and a set of natural-chemical compounds. The findings revealed that ACCA exhibited the highest binding affinity among the tested compounds, suggesting its strong interaction with the target protein. This information provides insights into the potential efficacy of ACCA and can guide further research and development efforts. Additionally, comparing the binding modes and interactions of other ligands contributes to our understanding of their SAR, facilitating the design of improved inhibitors for the target protein.

Molecular dynamics simulation

Molecular dynamics simulations (MDS) were conducted on the selected compounds, including 1-amino-cyclopropane-1-carboxylic acid (ACCA) and the 7Q3S protein, for a duration of 100 nanoseconds (ns). The aim was to assess the stability and quality of the protein–ligand complex throughout the simulation until convergence. The analysis of the simulation data revealed the root mean square deviation (RMSD) of the Cα-backbone of the 7Q3S protein bound to 1-amino-cyclopropane-1-carboxylic acid (ACCA). The RMSD plot, depicted in Figure 4A, showed that the Cα-backbone deviated by approximately 0.8 Å during the 100 ns simulation. This indicates that the complex remained relatively stable, with only minor fluctuations in the protein backbone. To evaluate the overall quality and convergence of the simulation, the relative mean squared deviation of the 1-amino-cyclopropane-1-carboxylic acid (ACCA)-bound protein was examined over the course of the 100 ns simulation. After 100 ns, the average difference between the reference structure and the final structure of the 7Q3S molecule was observed to be around two. Notably, the final structure of 7Q3S displayed significant deviations from the reference structure, particularly in residue positions 7–35, 58–95, and 198–219, as illustrated in Figure 4B. This suggests that the protein underwent structural rearrangements during the simulation. The protein's conformational changes upon ligand binding can

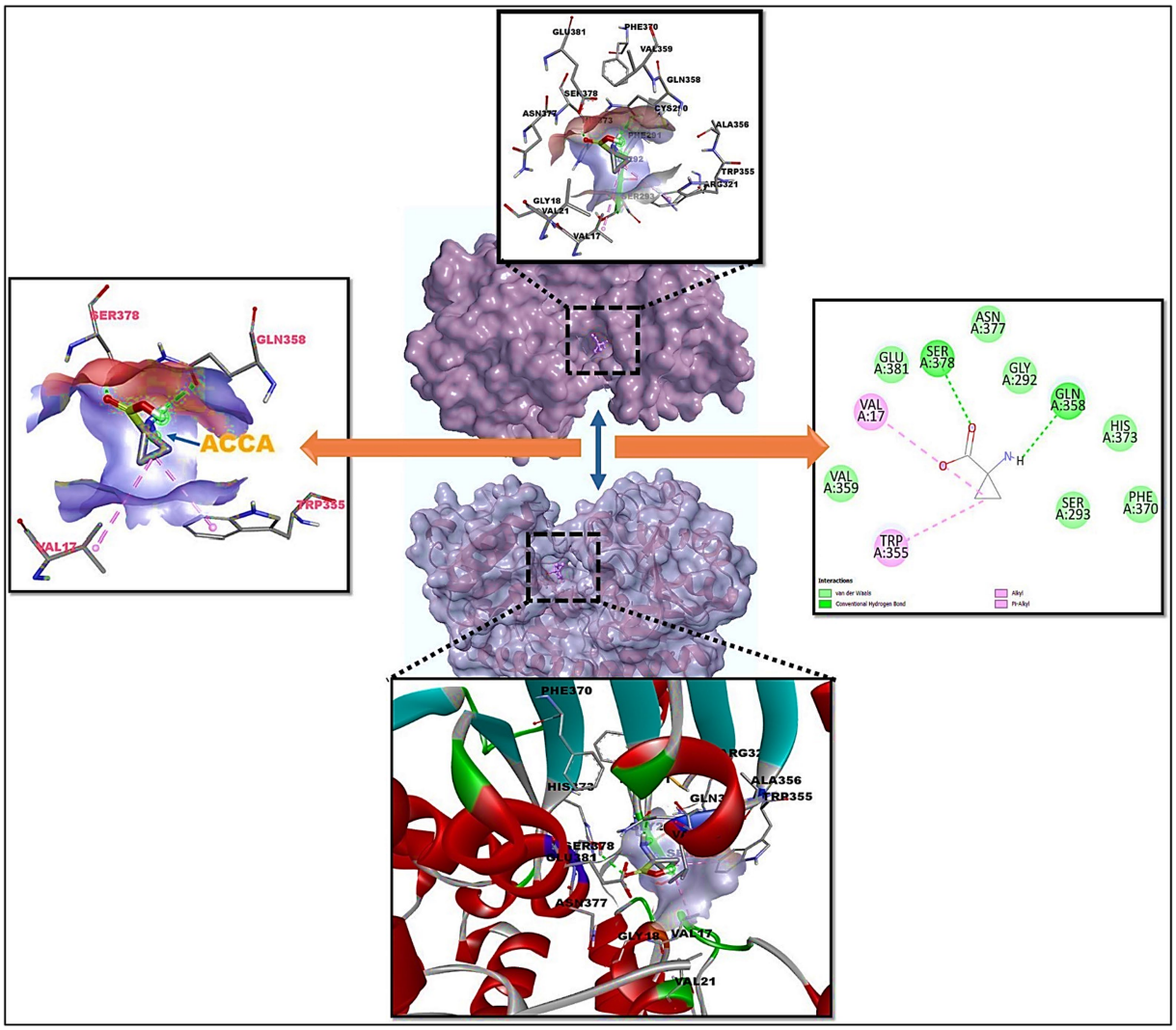


FIGURE 3
Molecular docking of 7Q3S bound 1-amino-cyclopropane-1-carboxylic acid (ACCA) and its 2D interaction diagram on right panel and ACCA's active residues on left panel.

be reflected in the radius of gyration (Rg), which characterizes the size and density of the protein. The Rg plot of the Cα-backbone, shown in Figure 4C, demonstrated that the 7Q3S protein exhibited Rg values ranging from 14.88 Å to 14.98 Å. This indicates considerable compactness of the protein–ligand complex, with an average change of approximately 0.7 Å in Rg throughout the 100 ns simulation. The formation and stability of hydrogen bonds between the 1-amino-cyclopropane-1-carboxylic acid (ACCA) ligand and the 7Q3S protein were also assessed. Figure 4D depicts that a single hydrogen bond was observed between ACCA and 7Q3S during the 100 ns simulation, indicating a favorable interaction between the ligand and the protein. Overall, the MD simulation successfully maintained the stability of the protein–ligand complex throughout the 100 ns duration. The minor fluctuations in the protein backbone, as indicated by the RMSD analysis, and the observed hydrogen bond formation demonstrate the overall stability and favorable interaction between ACCA and 7Q3S. These findings provide insights into the dynamic behavior and stability of the protein–ligand complex, contributing to a deeper understanding of the molecular mechanisms underlying the interaction and potentially guiding future optimization and design of ligands for improved binding and activity.

Strong hydrogen bonds were observed when 1-amino-cyclopropane-1-carboxylic acid (ACCA) interacted with the residues of 7Q3S that were predicted to bind ACCA. Additionally, various non-bonded interactions, such as hydrophobic contacts, ionic interactions, hydrogen bonding, and water bridges, were detected (depicted in Figure 5). The successful establishment of these interactions is crucial for forming a stable complex between the ligand and the protein. The combination of these diverse bonded and non-bonded interactions contributes to the overall stability of the complex formed between 1-amino-cyclopropane-1-carboxylic acid (ACCA) and the 7Q3S protein. This finding positions ACCA as a promising candidate for further investigation and potential applications as a bio-stimulant.

Figure 6 presents a ligand torsion map that provides insight into the structural changes occurring in each rotatable bond (RB) over the

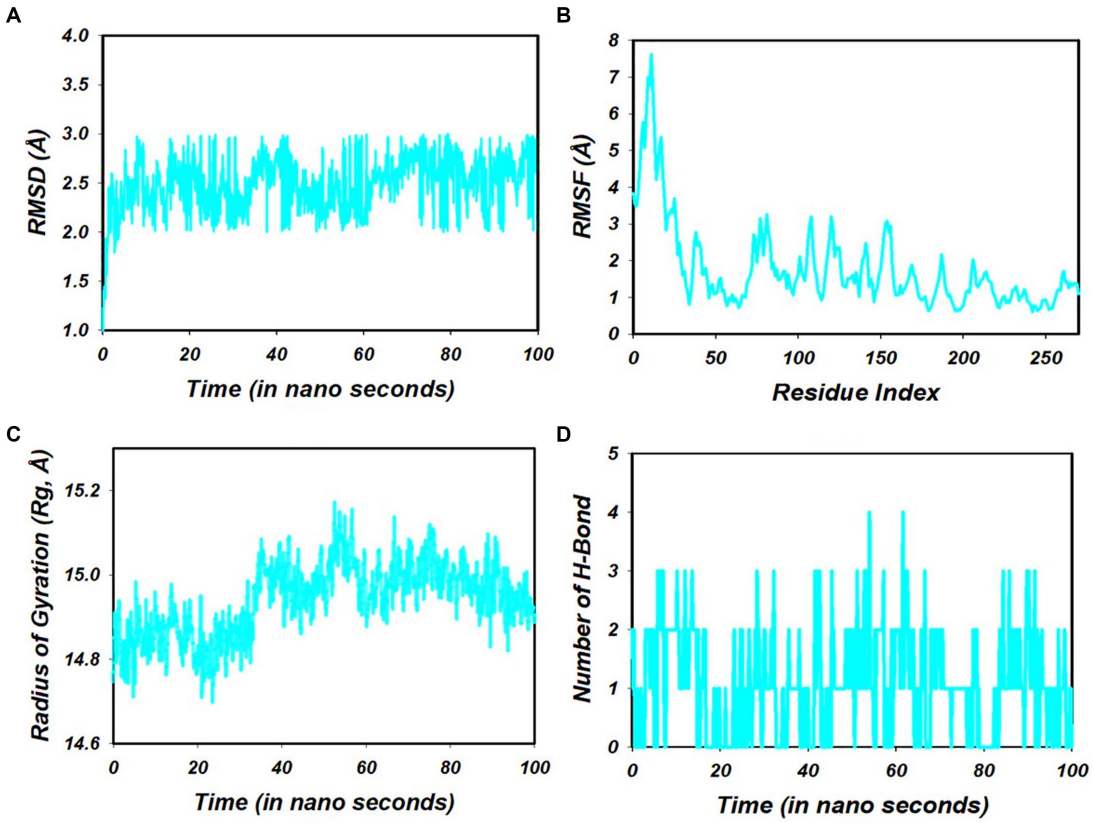


FIGURE 4
(A) RMSD of 7Q3S + 1-amino-cyclopropane-1-carboxylic acid (ACCA) after 100 ns run. **(B)** RMSF of 7Q3S + 1-amino-cyclopropane-1-carboxylic acid (ACCA) after 100 ns run. **(C)** Hydrogen bonding of 7Q3S + 1-amino-cyclopropane-1-carboxylic acid (ACCA) after 100 ns run. **(D)** Radius of gyration of 7Q3S + 1-amino-cyclopropane-1-carboxylic acid (ACCA) after 100 ns run.

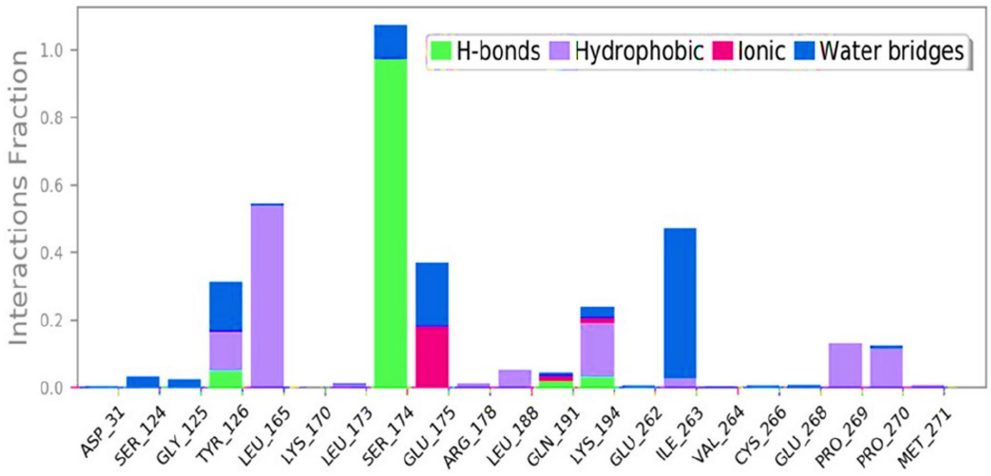


FIGURE 5
Various interactions formed in 100 ns simulation run.

course of the simulation from 0.00 to 100.00 ns. The top part of the figure displays a two-dimensional representation of the ligand's connections, which are capable of rotation. The presence of dial plots and bar plots in the same color indicates that a specific bond can undergo rotation. The simulation progresses in a clockwise direction around the screen, starting from the center of the screen. Dial plots and bar charts are employed to depict the probability distribution of the torsion angles. These plots allow us to visualize the different conformations adopted by the ligand as it undergoes rotations. During this type of analysis, it is crucial to closely examine the histogram,

torsion potential, and conformational strain of the protein. By doing so, we can determine whether the ligand maintains its bound shape throughout the simulation. Monitoring the stability and reliability of the ligand–protein complex is essential for understanding its behavior and assessing the impact of the ligand on the protein's function. Analyzing the ligand torsion map and observing the distribution of torsion angles provides valuable insights into the binding mode and the potential influence of the ligand on the protein's structure and dynamics. This information aids in assessing the stability and reliability of the ligand–protein complex and contributes to our understanding of the molecular interactions involved in ligand binding.

Discussion

In this in-depth study, we employed computational methods such as molecular docking and molecular dynamics simulations to critically analyze the interaction between 1-amino-cyclopropane-1-carboxylic acid (ACCA) and the protein 7Q3S. Our objective was to evaluate ACCA's potential as a bioactive compound to enhance the stress resilience and pathogen resistance of maize, a critical cereal crop in India's agricultural landscape.

Our molecular docking studies indicated that ACCA exhibited strong binding affinity to the protein 7Q3S, as suggested by a Gibbs free energy of −9.98 kcal/mol. A high binding affinity is an important determinant of a bioactive compound's effectiveness, suggesting the potential of ACCA to positively influence maize's resilience to stress and virulence against pathogens. The specific interactions between the ligand, 1-amino-cyclopropane-1-carboxylic acid (ACCA), and the target protein, 7Q3S, are a testimony to the intricate relationship between the biological activities and the molecular structures. It is this intricate relationship that can shed light on the function of ACCA in enhancing pathogen and stress resistance in *Zea mays*.

Among the various residues ACCA interacts with, Val17 and Trp355, which form pi interactions, are particularly significant. Pi interactions are critical for stabilizing the ligand–protein complex due to their unique ability to involve electron-rich aromatic systems, contributing to the overall binding affinity and stability of the

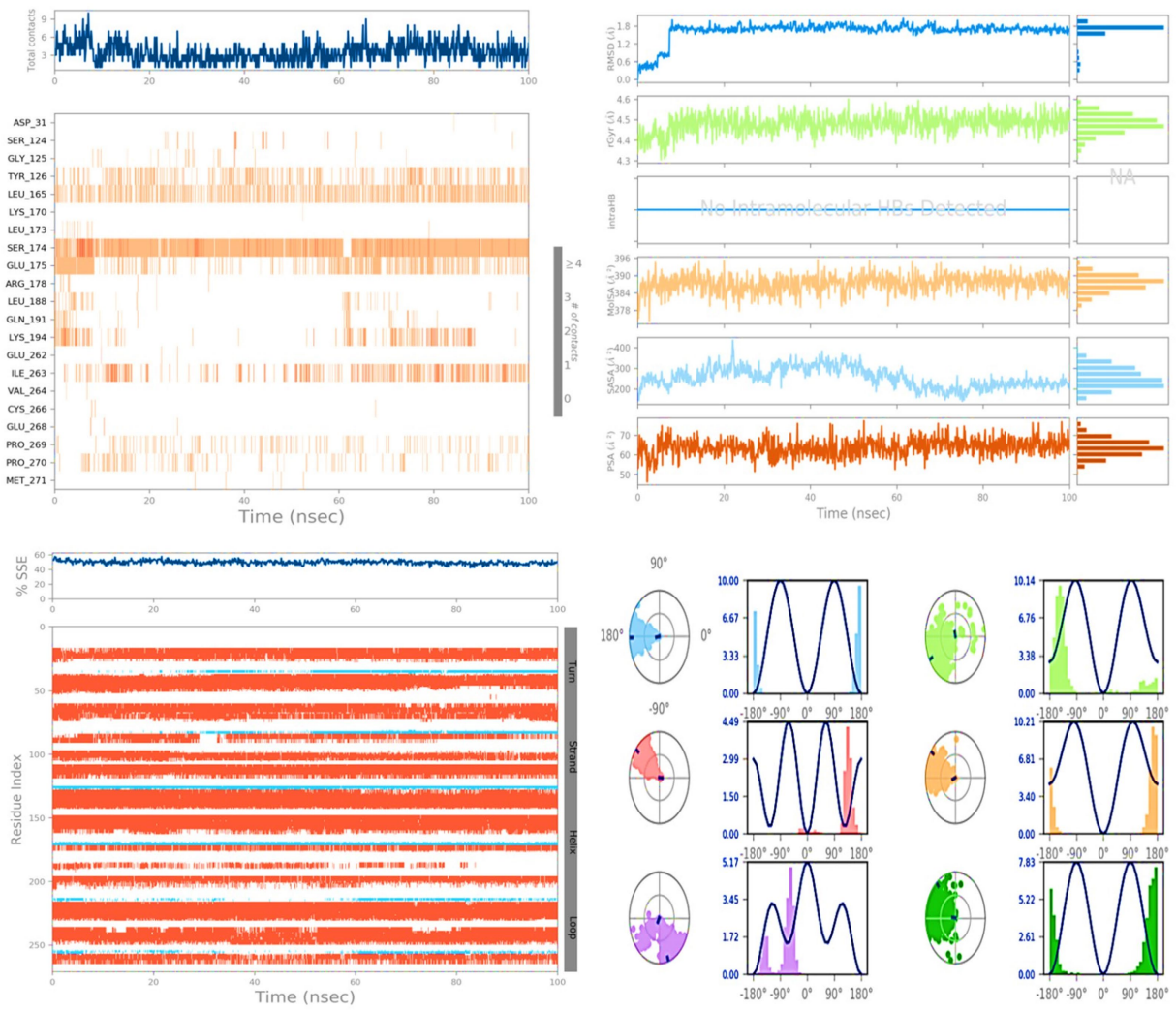


FIGURE 6
Illustration of protein ligand contacts, ligand interactions, proteins secondary structure and ligand torsion profile.

ACCA-7Q3S complex. On the other hand, a multitude of residues, including Glu381, Ser378, Asn377, Gly292, Gln358, His378, Phe370, Ser293, and Val359, are involved in van der Waals interactions with ACCA. These non-covalent interactions play an essential role in establishing the structural integrity of the ACCA-7Q3S complex and facilitating the proper orientation of ACCA within the protein's active site. They are essential for the recognition and selectivity of ACCA by 7Q3S and significantly contribute to the overall binding energy, potentially resulting in the observed effects on pathogen and stress resistance in maize.

Moreover, the identification of these specific residue-ligand interactions paves the way for future studies aimed at elucidating the exact molecular mechanisms through which ACCA exerts its effects. For instance, understanding whether these interactions lead to conformational changes in the protein, alter its enzymatic activity, or modulate its interaction with other proteins could provide critical insights into the mechanisms underlying ACCA-induced stress and pathogen resistance. This could lead to the design of more efficient bioactive compounds, leading to improved crop resilience against biotic and abiotic stressors.

Further studies, such as mutagenesis of the involved residues, could offer additional confirmation of the role of these specific amino acids in the observed effects, providing a more nuanced understanding of the molecular pathways involved in plant defense against pathogens and stress.

Further, we assessed the stability of the ACCA-7Q3S complex through molecular dynamics simulations over 100 ns. The findings pointed towards a stable complex with a constant radius of gyration, suggesting the possibility of a durable ACCA-7Q3S interaction. Importantly, this interaction was reinforced by hydrogen bonding, indicative of a robust complex formation. These findings underscore the potential of the ACCA-7Q3S complex as a plausible bioactive compound to enhance maize's resilience. Using ligand torsion map analysis, we gained valuable insights into the temporal conformational changes of the ACCA-7Q3S complex. Understanding these structural dynamics is integral to ascertaining the stability and potential efficacy of this bioactive compound. It is crucial to note that while our findings are based on robust computational methodologies, they serve as a complement to empirical validation, not a substitute. Future studies should prioritize *in vitro* and *in vivo* assays to verify our findings and further evaluate ACCA's potential in enhancing maize's resilience to environmental stressors and pathogens.

Our study provides compelling evidence for the potential of ACCA as a bioactive compound, underscored by its strong binding affinity and stable interaction with the 7Q3S protein. However, we acknowledge the need for extensive research to understand the molecular pathways through which ACCA enhances plant resilience. This knowledge could expedite the formulation of targeted strategies for crop protection and enhancement. Furthermore, our research accentuates the value of computational methodologies in identifying prospective bioactive compounds, an integral step in promoting sustainable agricultural practices and securing food production.

Conclusion

In the diverse ecosystem of *Zea mays*, various pathogenic microorganisms pose a formidable threat to the plant's health, significantly impacting the yield and quality of this crucial crop. Furthermore, challenges linked with low-irrigated soils, especially in drought-prone regions with sandy soil composition, pose an enduring hurdle to traditional farming methods. Amid such difficulties, rainfall variability and limited water availability further exacerbate the strain on the crops. In this context, our study reveals a promising avenue to alleviate these issues. We found that the application of compounds derived from both plant and Agrobacteria, especially 1-amino-cyclopropane-1-carboxylic acid (ACCA), could potentially mitigate the negative effects of intermittent soil drying, thereby safeguarding the health and vitality of maize cultivations. ACCA, in particular, displayed remarkable binding energy (−9.98 kcal/mol), highlighting its potential to induce virulence, bolster soil moisture retention, and improve the resilience of *Zea mays* under abiotic stress. More than just a novel discovery, this represents a paradigm shift in our approach to crop management, particularly for maize. By utilizing ACCA during the growth stages of maize plants, we can confer resistance against pathogenic infections and enhance crop yield. This potent compound emerged as a forerunner in our study, demonstrating potential to significantly augment virulence and stress tolerance in maize plants.

Our findings illuminate an exciting prospect for leveraging ACCA's potential to significantly bolster crop yield, quality, and resilience in *Zea mays*. However, the path forward demands comprehensive exploration. Future research should focus on delving deeper into ACCA's mechanistic pathway, understanding its optimal concentration for crop application, and exploring potential synergistic effects with other natural chemicals. Furthermore, real-world implementation studies would validate ACCA's efficacy and practicality under diverse agricultural conditions. Our findings provide a critical stepping stone towards a future of sustainable agriculture, marked by improved crop resilience, high yields, and enhanced food security.

Limitations of the study

Despite the promising outcomes of this research, it's crucial to recognize certain limitations that exist in our study and regard them as opportunities for further scientific exploration.

Computational predictions

While our study relied heavily on computational methodologies such as molecular docking and molecular dynamics simulations, these theoretical models might not completely emulate real-world biological environments. Therefore, translating these findings into practical applications requires careful and rigorous empirical validation.

Single compound focus

Our study primarily focused on 1-amino-cyclopropane-1-carboxylic acid (ACCA). Though ACCA has shown significant promise, the vast world of natural compounds remains largely unexplored. Future studies could expand upon our research by investigating other potential bioactive agents.

Limited environmental conditions

Our research predominantly revolved around conditions of drought and pathogenic stress. Other environmental stressors such as salinity, temperature extremes, and nutrient deficiency were not specifically addressed. Hence, subsequent research could seek to understand the role of ACCA under these varied stress conditions.

Detailed mechanistic pathway

While the study provided insights into the interaction between ACCA and 7Q3S protein, a comprehensive understanding of the exact mechanistic pathway involved in enhanced virulence and stress tolerance is yet to be fully elucidated.

In vitro and *In vivo* validation

The current study was computational in nature. Hence, *in vitro* and *in vivo* experiments are imperative to confirm the effectiveness of ACCA and its impact on maize plant health and yield under practical conditions.

Taken together, these limitations do not diminish the value of our findings, but instead offer a fertile ground for future research and potential breakthroughs. As we continue to refine our understanding of ACCA and its potential applications, we move closer to the goal of promoting sustainable agricultural practices and ensuring food security.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

SD and AS: conceptualization, investigation, supervision, data curation, formal analysis, resources, writing—original draft, and writing—review and editing. AE, AB, RE, MV, PT, SW and LSW: data analysis, investigation, manuscript preparation, and review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Marvels of Bacilli in soil amendment for plant-growth promotion toward sustainable development having futuristic socio-economic implications

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Microorganisms are integral components of ecosystems, exerting profound impacts on various facets of human life. The recent United Nations General Assembly (UNGA) Science Summit emphasized the critical importance of comprehending the microbial world to address global challenges, aligning with the United Nations Sustainable Development Goals (SDGs). In agriculture, microbes are pivotal contributors to food production, sustainable energy, and environmental bioremediation. However, decades of agricultural intensification have boosted crop yields at the expense of soil health and microbial diversity, jeopardizing global food security. To address this issue, a study in West Bengal, India, explored the potential of a novel multi-strain consortium of plant growth promoting (PGP) *Bacillus* spp. for soil bioaugmentation. These strains were sourced from the soil's native microbial flora, offering a sustainable approach. In this work, a composite inoculum of *Bacillus zhangzhouensis* MMAM, *Bacillus cereus* MMAM3), and *Bacillus subtilis* MMAM2 were introduced into an over-exploited agricultural soil and implications on the improvement of vegetative growth and yield related traits of Gylcine max (L) Meril. plants were evaluated, growing them as model plant, in pot trial condition. The study's findings demonstrated significant improvements in plant growth and soil microbial diversity when using the bacterial consortium in conjunction with vermicompost. Metagenomic analyses revealed increased abundance of many functional genera and metabolic pathways in consortium-inoculated soil, indicating enhanced soil biological health. This innovative bioaugmentation strategy to upgrade the over-used agricultural soil

through introduction of residual PGP bacterial members as consortia, presents a promising path forward for sustainable agriculture. The rejuvenated patches of over-used land can be used by the small and marginal farmers for cultivation of resilient crops like soybean. Recognizing the significance of multi-strain PGP bacterial consortia as potential bioinoculants, such technology can bolster food security, enhance agricultural productivity, and mitigate the adverse effects of past agricultural activities.

KEYWORDS

sustainable agriculture, novel consortium of *Bacillus zhangzhouensis*, *B. subtilis*, *B. cereus*, multi-strain PGP bacterial inoculant, microbe-assisted bioaugmentation, plant-growth enhancement, resident bacterial community modulation

1 Introduction

Intensification of crop production through extensive use of agrochemicals has been the major driving force for a quantum leap in crop yield after the green revolution. Over-exploitation of arable lands to feed the rapidly growing world population has negatively impacted the structure and function of soil by depleting nutrient levels, lowering microbiological diversity, and crop productivity (Huang et al., 2019) posing a serious threat to global food security. The damaged soils fail to regain their fertility satisfactorily and are unable to regenerate naturally (Goenster et al., 2017). Soils contain a myriad of microorganisms which constitute a characteristic microbiome playing a pivotal role in functioning of ecosystem and thereby, maintaining soil health (Maron et al., 2018). Research works spanning over the last few decades have established the importance of beneficial soil microbes, which can directly influence plant growth through mobilization of soil nutrients, secretion of plant beneficial secondary metabolites (such as phytohormones, siderophore) as well as indirectly protect plants from biotic and abiotic stresses, producing various stress metabolites (Bhattacharyya and Jha, 2012; Gouda et al., 2018).

In the rapidly growing sectors of sustainable agriculture, beneficial soil microorganisms (BSM) are assumed to steer the bio-based revolution in the near future as a potential alternative to complement or replace chemical fertilizers and pesticides (Malusà et al., 2021; Manfredini et al., 2021). Soil microbiome based approach has emerged as a promising strategy to mitigate the problem of soil productivity depletion, in an eco-friendly way (O'Callaghan et al., 2022). A healthy microbiome can be established in the crop field through the introduction of plant growth-promoting microbes (PGM) which exert their plant-favorable activity after building up a critical amount of biomass into the soil (Vassileva et al., 2020). These modulations may alter plant performance and soil health, and thereby, inducing unpredictable feedback reactions (Berg et al., 2021). It is well established that PGM having high enzymatic activity, phytohormone, and osmolytic metabolite-producing potential are effectively involved in plant health promotion and nutrient mobilization (Haas and Keel, 2003; Shilev et al., 2019). A recent study conducted by Kumar et al. (2023) on wheat rhizosphere in Upper-Gangetic plain, showed that various soil factors and conditions (e.g. available nitrogen, potassium, organic carbon) can be effectively improved through appropriate microbiome manipulation strategies.

Furthermore, some natural products (like vermicompost, agro-industrial wastes, etc.) can contribute to improving microbial diversity and promotes the growth of indigenous soil microbes within the soil–plant system (Arancon et al., 2006; Strachel et al., 2017). Implementation of organic farming through successful utilization of PGP microbes, protects and preserves soil health through sustainable and eco-friendly crop management practice by conservation and restoration activities (Gamage et al., 2023). Most of the studies in this field have revolved around utilization of single-strain microbial inoculants having multifarious plant growth promoting (PGP) activity. A recent study Hu et al. (2021) suggested a unique strategy of designing inoculants to rejuvenate the resident beneficial soil microbes (BSM) already present in the rhizosphere, using multi-strain microbial inoculants having multiple PGP traits. The effect of the introduced microbial inoculants may be transient or prolonged in the soil and it is dependent on the diversity of autochthonous soil microbial communities (Mallon et al., 2015; Mawarda et al., 2020). However, the potential of multi-strain residual bacterial consortia as an effective microbial inoculant to boost up crop production in long-term used nutrient depleted agricultural soil, is yet to be properly explored, especially in West Bengal, India. In this context, the objective of the present investigation was to explore the implications of soil amendment with a novel consortium of three residual PGP *Bacillus* spp., pre-isolated from the resident microbial flora of an over-exploited land. Vermicompost was used as a natural additive along with the multi-strain bacterial inoculant during soil augmentation. The soil collected from the same sampling field from where PGP *Bacillus* spp. were isolated, was used to carry out the whole study. Implications of this soil augmentation practice on the improvement of vegetative growth and yield related characteristics of *Gylcine max* (L) Meril. plants were evaluated, growing them as test plant, in pot trial condition. Furthermore, the potentiality of the present strategy to improve the resident bacterial community health of the nutrient-depleted soil, was also investigated.

Abbreviations: UNGA, United Nations General Assembly; PGP, Plant growth promoting; SDG, Sustainable developing goal; BSM, Beneficial soil microorganisms; IAA, Indole acetic acid; GA, Gibberellic acid; ICAR, Indian Council of agricultural research; WAE, Weeks after seedling emergence.

2 Materials and methodology

2.1 Sampling site and collection of soil sample

The collection of soil samples was conducted from a nutrient-depleted arable land located at Bahadurpur, South 24 Parganas District, West Bengal, India, (21^0 26' N-22^0 to 38' N, 87° 57′ to E-89° 09′E), abiding by the protocol of TNAU (2013).[1] The soil was fine-loamy, Aeric-Epiaquerts type (Khan and Kar, 2020). Soil nutrient status like, organic (SOC) carbon, and available nitrogen (N), phosphorus (P), and potassium (K) content was estimated using standard methods as followed by Jackson (1967) and Mandal et al. (2020). For metagenomic analysis, fresh soil samples from the field and pots of each experimental set-up (at the fruit harvesting stage of soybean plants) were collected and pooled separately. Collected soil samples were stored separately at-20°C refrigerator until DNA extraction. For conducting pot experiments freshly procured non-sterilized soil from the sampling field was used.

2.2 Selection of PGPB strains and bacterial inoculant formulation

Three potent pre-isolated plant-growth promoting bacteria (PGPB) strains, identified by 16S rRNA sequencing as *Bacillus zhangzhouensis* MMAM (Accession no. MT 185655), *Bacillus cereus* MMAM3 (Accession no. MT 730003), and *Bacillus subtilis* MMAM2 (Accession no. MT 72561.1), were used as a novel consortium in this work. They were isolated from the resident microbial flora of the over-exploited soil, used in this investigation, and reported to have N-fixing, P-solubilizing, phytohormone Indole acetic acid (IAA), Gibberellic acid (GA) producing, and siderophore secreting ability. Additionally, these isolates can produce antimicrobial metabolites such as, amylase, protease, catalase, peroxidase, ammonia, and hydrogen cyanide (Mukhopadhyay, 2022). Fresh broth cultures of each of the isolates were prepared separately (HiMedia) incubating them in inoculated Luria Bertani (LB) (HiMedia) media for 48 h(h) at 37° C in an orbital shaker at 160 rpm. The cell counts of the freshly grown bacterial suspensions were adjusted to 4.5×10 mL^{-1} (per milliliter). The microbial consortium suspension contained each of the three individual bacterial cultures at a 1:1:1 ratio. The inoculant formulation constituted 20% of the broth culture of the consortium, 30% sterilized distilled water, 30% sunflower oil, and 20% Tween-80 (Sigma. P6224).

2.3 Evaluation of *in vivo* growth-promotion efficacy of the bacterial consortium

The pot trial experiment was designed to assess *in vivo* plant growth promotion ability of the amendments in controlled conditions. *Glycine max* Meril. var. JS-0335 was used as the test plant. The seeds were procured from the ICAR-Indian Institute of Soybean Research, Indore. The non-sterilized, freshly collected soil from the field was used for this work. Seeds were surface sterilized using sodium hypochlorite solution (0.1%) for 5 min and were then rinsed thoroughly with sterile distilled water for 5 times before sowing in pots.

2.3.1 Experimental design

The pot trial experiment was carried out in polythene bags of 28 centimeter (cm) X 26 cm X 26 cm dimension, each containing 5 kg of freshly procured non-sterilized field soil. The bags of each treated and untreated set-up were maintained in four replicates, in open air condition. In the amendment, vermicompost, procured from Nimpith Krishi Vigyan Kendra (West Bengal), was applied to the pot @ 100 gram per kilogram (kg^{-1}) of soil. Details of the experimental design are furnished below:

Experimental set-up 1. SU: untreated field soil.

Experimental set-up 2. SU: freshly collected field soil + vermicompost.

Experimental set-up 3. SBC: freshly collected field soil + multi-strain bacterial consortium.

Experimental set-up 4. SVBC: freshly collected field soil + vermicompost + consortium.

The topsoil was covered with coco peat (1.5 cm layer) and six seeds were sown randomly in each pot. Twenty ml of the first dose of inoculant formulation was applied to the bags of the respective treated set-up near the rhizospheric region of the plants, 15 days after the seedling emergence stage, followed by two successive doses at 35- and 55-day stages, respectively. An equal amount of water was applied to each pot on every 2 days. De-weeding was practiced once in a week.

2.3.2 *In vivo* plant growth promotion study

The efficacy of the amendments was tested based on their impact on selected growth characteristics of the potted plants and their yield related performance. Data were recorded every 4, 8, and, 12 weeks after the seedling emergence stage (WAE) for analyzing vegetative parameters of plants like the total number of leaves, leaf area, plant height, and the number of root nodules plant^{-1}. The first onset of flowering (days) and the total number of pods plant^{-1} were recorded. After harvesting, the number of seeds pod^{-1}, and the dry weight of 100 seeds were kept in record.

Chlorophyll (chl) content of leaves such as chl-a, chl-b and total chl (a + b) were measured spectrophotometrically at 4, 8, and 12 WAE, respectively. To extract chlorophyll pigment, 80% acetone was used. Freshly collected mature leaves (2nd and 3rd leaves) from the pot-grown soybean plants were used (Liang et al., 2017). The chl-a, chl-b and total chl content were estimated according to the equation of Arnon (1949):

$$\text{Chlorophyll a}\left(\mu g / \text{mL}\right) = 12.7\left(\text{A}_{663}\right) - 2.69\left(\text{A}_{645}\right).$$

$$\text{Chlorophyll b}\left(\mu g / \text{mL}\right) = 22.9\left(\text{A}_{645}\right) - 4.68\left(\text{A}_{663}\right).$$

$$\text{Total chlorophyll}\ \left(\mu g / \text{mL}\right) = 20.2\left(\text{A}_{645}\right) + 8.02\left(\text{A}_{663}\right).$$

2.3.3 Statistical analyzes

Python software version 3.11+ and its modules along with scientific computation libraries were used for plotting, analyzing, and

1 https://agritech.tnau.ac.in/agriculture/agri_soil_sampling.html/TNAU-2013

visualizing the data obtained during the pot trial experiment. We performed two-way ANOVA on the vegetative and one-way ANOVA for reproductive parameters and subsequently conducted Tukey's *Post Hoc* test to investigate the significant differences in the means of the observed characteristics. Both the ANOVA and Tukey's test were conducted at a standard significance level of 5%. Normality test was performed to determine, if the concerned variables follow normal distribution pattern or not. Based on it, paired sample t-test was conducted to detect, whether the effect of combined treatment of vermicompost and bacterial consortia on different plant parameters were significant or not. To study the effect of the combined treatment of vermicompost and bacterial consortia on vegetative growth and yield related characteristics of soybean plants, the observed plant parameters in SVBC condition were compared with those of SU condition by applying paired t-test. Finally Logistic Regression mode was followed to find out if the improvement in vegetative parameters of plants such as, total no. of leaves plant^{-1} (X1), leaf area (X2), total chl content of leaves (X3) and total no. of root nodules, is reflected on the yield characteristics, which is captured best by total no of pods plant^{-1}. Total no. of pods plant^{-1} (Yi) is the dependent binary variable. The empirical specification is:

$$Yi = \alpha + \beta 1\, X1 + \beta 2\, X2 + \beta 3\, X3 + \beta 4\, X4 + \text{error term.}$$

Where:
Yi = 0, at vegetative state,
Yi = 1, at harvesting state.

2.4 DNA extraction and metagenomic sequencing of treated and non-treated field soil

The MOBIO PowerSoilTM DNA Isolation kit (Qiagen, United States) was utilized for the isolation and extraction of bacterial DNA from sieved soil samples for the execution of downstream metagenomic analyzes (Bag et al., 2016; Ghosh et al., 2022). The DNA hence obtained was sequenced on Illumina MiSeq using reagent kit V3 according to the manufacturer's protocol for generating 2 × 300 bp paired-end reads and quality assessment was carried out using Nanodrop followed by semi-quantitative estimation of DNA via agarose gel electrophoresis. QUBIT assay was performed to obtain the precise concentration of the extracted DNA. Gene library preparation was carried out by amplifying the standardized V3-V4 region of 16SrRNA as per Illuimina gene library construction protocol.

2.5 Bioinformatics analysis/analyses

The sequenced raw reads were processed through the FASTQC pipeline for quality checking followed by which the screened sequences surpassing the quality threshold were finally assembled via homopolymer elimination, minimization of artefactual noise and probable contamination, using SILVAngs (1.3) pipeline (Lepinay et al., 2018). In accordance with the pipeline followed by Ganguli et al. (2017) and Mukhopadhyay et al. (2021). Operational Taxonomic Units (OTUs) were clustered using QIIME2, and microbial abundances were analyzed using KRONA charts (Estaki et al., 2020). The user-end reads yielded from Illumina sequencing were used as query sequences and subjected to the LAST algorithm for matching against the RDP_16S_18S database, for analyzing bacterial matches, at different taxonomic levels, using an alignment score cut-off of 0.8 subsequent to the elimination of reads having very high e-values. The data obtained herein was used for downstream analyzes. Starting from the widest taxonomic level, it assigned a taxonomic label to each read. The taxon that received the most hits was used for this purpose. The analysis continued until a confidence level was reached or numerous taxa were supported by the same quantity of high-quality hits. All unmatched or unclassified reads were removed from the data for downstream analyzes.

Using Krona Tools, the representative taxon was displayed in interactive graphs (Ondov et al., 2011). Data on bacterial and archaeal members were identified by using PATRIC (Wattam et al., 2017). Identification of common elements among the experimental soil under differential treatment conditions was done using Venny 2.1.0 to generate Venn Diagrams (Oliveros, 2015). An in-house algorithm has been used to integrate microbial co-inhabitance patterns and several updated datasets of different curated microbial function maps. The common genera among datasets of the field soil (S) were compared with that of soybean plant-grown untreated (SU), vermicompost-treated (SV), consortia-treated (SBC), and jointly vermicompost-consortia-treated (SVBC) soil. The results thus obtained were used to generate the Venn diagram. The enriched metabolic pathways were determined according to the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000). The most crucial functions were examined, and literature was mined to corroborate the data obtained as well as to correlate it with subsequent findings, including the most significant genera (as well as their inherent hierarchy) in the dataset were visualized as heatmaps, showing variations across the samples under study. The predominant functions in metabolic participation were also visualized and separated using enrichment networks. Finally, abundant genera across the samples were represented through a clustering algorithm using R code (Kolde, 2019).

3 Results and analysis

3.1 Soil nutrient status

Soil parameters like organic carbon, available potassium, nitrogen, and phosphate content were estimated, and the results are presented in Table 1. The soil was observed to have a very low level of organic carbon (0.34%) and available nitrogen (48.7 mg/kg) content according to the Indian standard (Khurana and Kumar, 2022). The estimated levels of available phosphorus and potassium were recorded as 27.25 mg/kg and 136.60 mg/kg, respectively.

All values expressed as Mean ± Standard deviation.

3.2 Evaluation of *in vivo* growth-promotion efficacy of the PGPB consortium

The influence of the soil amendment on the vegetative growth, flowering, and yield-related behavior of soybean plants in different experimental conditions (SU, SV, SBC, and SVBC) was analyzed. Varying effects were observed at 4, 8, and 12 WAE stages of the plant growth with respect to vegetative growth characteristics like leaf

density, leaf area, plant height, and nodule numbers. The non-inoculated plants grown in the SU set-up were inferior with respect to most the parameters studied, whereas the highest improvement was recorded in bacterial consortium-inoculated plants grown in vermicompost--treated soil (SVBC) followed by SBC condition (Figure 1).

The findings of vegetative growth-related traits of the plants at different experimental conditions are furnished in Figure 2. The total number (mean) of leaves plant^{-1} observed, at 12 WAE stages in SU, SV, SBC, and SBVC set-ups were 22, 34, 43, and 51, respectively (Figure 2A). Leaf area (mean) increased remarkably in all the treated experimental set-ups at stages over to that of the un-inoculated control condition, showing most striking improvement in at the 12 WAE stage of the plants, ranging from 40.2 sq. cm in SU to 61.7 sq. cm, 99.8 sq. cm, and 102.5 sq. cm in SV, SBC, and SVBC conditions, respectively (Figure 2B). Enhancement in plant height was evidenced by 14.13, 21.73, and 32.6% increase in the SV, SBC, and SVBC set-ups at 12 WAE, and a similar trend was recorded at 4 and 8 WAE stages (Figure 2C). The root nodule number was significantly high in SBC and SBVC set-ups in comparison to the other set-ups (Figures 2E,F). Total chlorophyll content leaves showed a significant increase by 24.6%. 25.6%, and 55.4%, respectively under SV, SBC, and, SVBC conditions, at 12 WAE stage over to that of the untreated one (Figure 2G). The overall trend indicates an enhancement in plant performance following soil amendment with only vermicompost, only bacterial inoculant and a combined treatment with vermicompost-consortium. However, the most promising result was observed in SBVC condition.

The pot trial experiment showed a significant improvement in reproductive and yield-related traits of the soybean plants following amendment practices (Figure 3). Soil augmentation with the joint-treatment of vermicompost and the novel consortium, exerted a positive effect on the initial blossoming stage as evidenced by 8.1, 16, and 20.4% decrease in the first onset of flowering days in SV, SBC, and SVBC in comparison to that of SU set-up indicating early flowering in consortium treated conditions (Figure 3A). Combined application of vermicompost and bacterial consortium inoculant tremendously influenced the number of pods node^{-1} (Figure 3B) and consequently, on the total number of pods plant^{-1}, which was highest in SVBC condition (88.1% increase) compared to the SU set-up (Figure 3B). An increase in the number of seeds pod^{-1} was also evident in inoculant-treated soil, both with and without vermicompost supplementation conditions (Figure 3C). Application consortium incredibly increased the dry weight of seeds by 24.46 and 45.7% in SBC and SBVC set-ups, respectively whereas in vermicompost-enriched soil, it improved by only 7.4% (Figure 3D).

TABLE 1 Nutrient status of soil sample.

Soil parameter	Result
Organic carbon content (%)	0.34 ± 0.416
Available nitrogen (mg/kg)	48.7 ± 0.135
Available phosphorus (mg/kg)	27.25 ± 0.057
Available potassium (mg/kg)	136.60 ± 0.226

All values expressed as Mean ± Standard deviation.

FIGURE 1
Effect of bacterial inoculum on the growth of *Glycine max* plants in different experimental set-ups. **(A)** Vegetative growth pattern of plants at 8 weeks after seedling emergence stage in treated and untreated set-ups; **(B)** A portion of twigs of untreated and treated plants show fruiting behavior.

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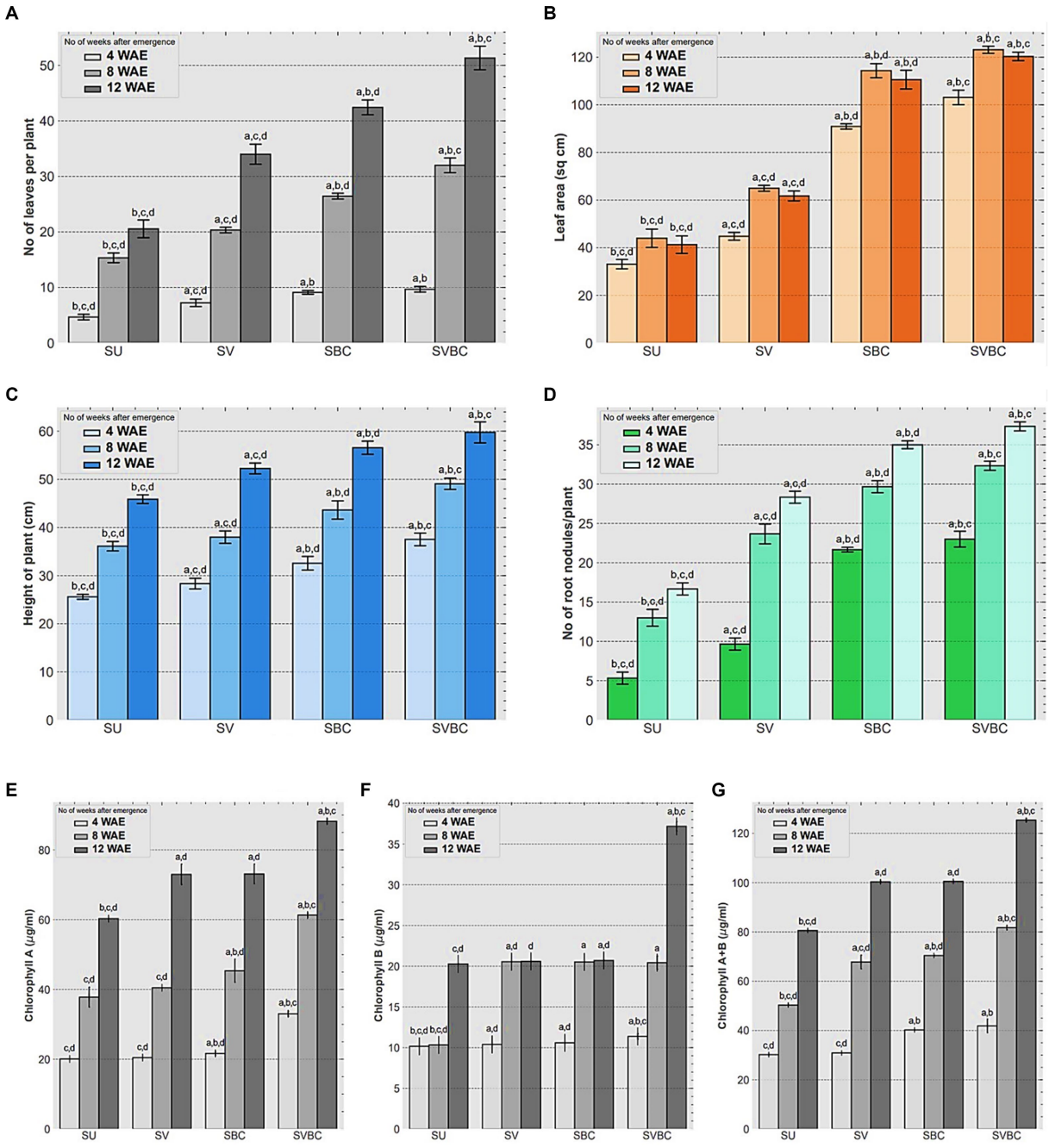

FIGURE 2
Effect of different treatments on vegetative parameters and chlorophyll content of leaves of *Glycine max* Merill. plants at 4, 8, and 12-week stages after seedling emergence (WAE). **(A)** Number of leaves per plant; **(B)** Leaf area; **(C)** Height of plants, **(D)** Number of root nodules per plant; **(E)** Chlorophyll-a content of leaves; **(F)** Chlorophyll-b content of leaves; **(G)** Total Chlorophyll content of leaves. SU, untreated soil; SV, soil amended with vermicompost; SBC, treated with the bacterial consortium; SVBC, soil amended with vermicompost and bacterial consortium. Columns represent the mean values of the data for each characteristic and the error bars represent the standard deviation. Different letters on columns imply the significant difference between the means of the data ($p < 0.05$) as evaluated by Tukey's HSD test after a one-way ANOVA test.

During statistical analysis, Tests of Normality showed that, all the concerned variables related to plant growth and performance followed normal distribution as confirmed by Shapiro–Wilk test. The test statistics were not significant at 5% level (Table 1; Supplementary Table S1).

Hence, we proceeded for t-test which is appropriate for this study. It was observed that t-statistic was highly significant at 5% level which confirmed that there has been significant improvement in the total no. of leaves plant^{-1}, total no. of pods plant^{-1}, total no. of chlorophyll content of leaves, total no. of root nodules plant^{-1}, and dry weight of 100 seeds in SVBC condition over to that of SU condition (Supplementary Table S2). Finally, Logistic regression model indicates that, if there is per unit rise in the no. of leaves plant^{-1} (X_1), leaf area (X_2), total chl content of leaves (X_3) and total no. of root nodules, then

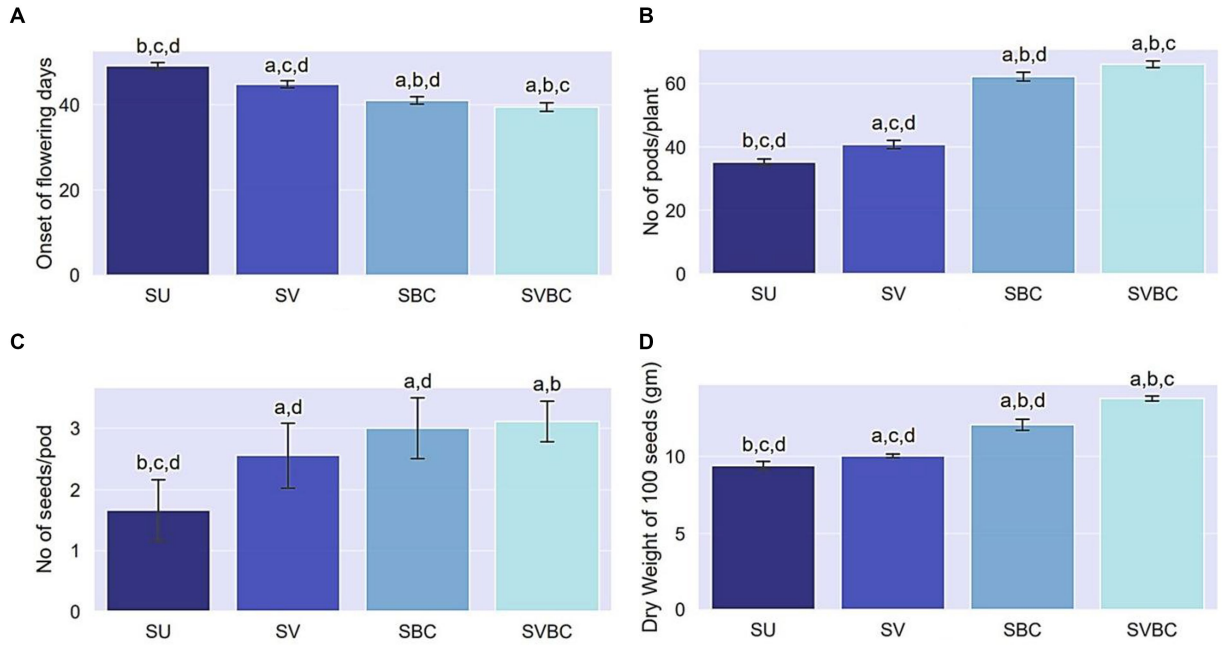

FIGURE 3
Effect of different treatments on reproductive and yield attributes of *Glycine max* Merill. Plants. **(A)** First onset of flowering (days); **(B)** No. of pods plant^{-1}; **(C)** No. of seeds pod^{-1}, **(D)** Weight of 100 seeds. Columns represent the mean values of the data for each characteristic and the error bars represent the standard deviation. Different letters on columns imply the significant difference between the means of the data (p<0.05) as evaluated by Tukey's HSD test after a one-way ANOVA test. SU, untreated soil; SV, soil amended with vermicompost; SBC, treated with the bacterial consortium; SVBC, soil amended with vermicompost and bacterial consortium.

TABLE 2 Logistic regression.

Step 0	Variables		Score	df	Sig.
		No_leaves	17.913	1	0.000
		Leaf_area	17.869	1	0.000
		Chl_content	17.984	1	0.000
		Root_nodules	17.631	1	0.000
	Overall statistics		17.988	4	0.001

there is likelihood that the no. of pod plant^{-1} will increase by 17.91, 17.86, 17.98, and 17.17.99 units, respectively (Table 2).

3.3 Insights into bacterial abundance in treated and untreated soil

The comprehensive analysis of our investigation was centered around field soil samples obtained from five unique environmental conditions. Each specific condition or environment was meticulously segregated and processed as an individual dataset, with the detailed specifications of each outlined in Table 3. For researchers and practitioners who are keen on delving deeper into the methodological specifics and ensuring data integrity, the Quality Control (QC) Parameters associated with the Paired-End Miseq Illumina Sequences for these datasets can be perused in Supplementary Table S3.

Upon an initial examination of the microbial diversity profiles across the datasets, certain patterns and disparities come to the fore. Most notably, the 5th dataset, designated as SVBC (an amalgamation of field soil that was treated with the combined efforts of vermicompost, bacterial consortia, and *in situ* soybean plants), presented an intriguing dichotomy. On the surface, its alpha diversity metric–a fundamental measure that encapsulates the richness and diversity within a singular ecosystem–was observed to be slightly subdued when juxtaposed with the 4th dataset. This might lead one to hastily surmise that SVBC was less diverse or less robust than its counterparts. However, a more nuanced metric, the Shannon diversity index, paints a contrasting picture. This index, which serves as a reliable barometer for the species diversity within an ecosystem, recorded a higher value for the SVBC dataset, as captured in Table 4. Such an observation insinuates that the SVBC environment, while potentially having fewer genera than others, exhibited a broader and more uniform distribution of those genera present. Such a balanced and evenly spread microbial community could be indicative of a robust and harmonized ecosystem. It implies that the SVBC condition may host a microbial environment that, while not the most varied, is characterized by stability, resilience, and an intricate balance of microbial interactions.

This interplay and uniformity might have implications for soil health, plant growth, and overall ecosystem stability, warranting further in-depth studies and explorations into the underlying mechanisms and benefits.

3.3.1 Dataset 1: soil sample code: S

The Krona map yielded by running the raw sequence reads through a suitable pipeline revealed that Actinobacteria was the most abundant phylum, followed by Chloroflexi, Firmicutes, Proteobacteria, and Acidobacteria in soil sample (S). Upon further screening of the

TABLE 3 Sample nomenclature codes and NCBI SRA accession number.

Data set	Sample code	Experimental condition	Analysis code	NCBI SRA Project: PRJNA689214
				SRA accession number
1	S	Field Soil	SAM3	SRX 9768638
2	SU	Field Soil+Soybean Plant	SAM2	SRX 9815238
3	SV	Field Soil + Vermicompost + Soybean Plant	DHB3	SRX 19133782
4	SBC	Field Soil + Bacterial Consortium+Soybean Plant	DHB1	SRX 19133762
5	SVBC	Field Soil+Vermicompost +Bacterial+Sybean plants	DHB2	SRX 19133763

Direct link to the data submitted to NCBI SRA: https://www.ncbi.nlm.nih.gov/sra/PRJNA689214.

TABLE 4 Alpha and Shannon diversity indices.

Data set	Sample code	Alpha diversity	Shannon diversity index
1	S	228	2.418
2	SU	368	3.142
3	SV	281	3.439
4	SBC	377	3.426
5	SVBC	328	3.496

putative top 10 genera that were most abundant in the given sample, *Arthrobacter* and *Streptomyces* had relative abundances of 15.58 and 10.79% (Figure 4).

3.3.2 Dataset 2: soil sample code: SU

It was found that Actinobacteria was the most abundant phylum, followed by Chloroflexi, Firmicutes, Proteobacteria, and *Acidobacteria*. The untreated field soil sample in the presence of soybean plants (SU) contained the following top 4 genera: *Arthrobacter* and *Streptomyces* (8%), *Rhodococcus* (4%), *Mycobacterium* (3%) and *Bacillus* (2%). Upon further screening of the putative top 10 genera that were most abundant in the given sample were *Arthrobacter* and *Streptomyces* had relative abundances of 13.15 and 11.92% (Figure 5).

3.3.3 Dataset 3: soil sample code: SV

The Krona map revealed that Actinobacteria appeared to be the most abundant phylum, followed by Acidobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, and Proteobacteria in the soil sample SU. It included the following top 4 genera: Gp6 (5%), *Gaiella* (3%), *Gemmatimonas* (2%), and Gp10 (2%) (Figure 6).

3.3.4 Dataset 4: soil sample: SBC

Proteobacteria was observed to be the most abundant phylum, followed by Acidobacteria, Actinobacteria, Planctomycetes, Firmicutes, Chloroflexi, and Bacteroidetes in soil sample SV. It contained of the following top 4 genera: Gp6 (6%), *Bacillus* (3%), *Gemmatimonas* (2%), and *Gaiella* (2%). Upon further screening of the putative top 10 genera recorded to be the most abundant in the given sample, *Gp6* and *Bacillus* have relative abundances of 15.21 and 5.63% (Figure 7).

3.3.5 Dataset 5: soil sample code: SVBC

The Krona map revealed that Proteobacteria was the most abundant phylum, followed by Acidobacteria, Actinobacteria, Firmicutes, Planctomycetes Chloroflexi, Bacteroidetes, and Verrucomicrobia. The soil sample SBC contained the following top 4 genera: Gp6 (6%), *Bacillus* (3%), *Gemmatimonas* (2%), and *Sphingomonas* (2%). Upon further screening of the putative top 10 genera that were most abundant in the given sample, Gp6 and *Bacillus* have relative abundances of 11.64 and 7.51% (Figure 8).

3.4 Comparative profiling of treated and untreated soil samples

Some of the bacterial genera common across all the datasets spanning varying ranges of treatments were *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microbacterium*, *Paenibacillus*, *Bacillus*, and *Pseudomonas*. The bacterial genera unique to the untreated field soil sample were *Neptuniibacter*, *Lysinimonas*, *Alcanivorax*, *Campylobacter*, *Neisseria*, *Methylococcus*, and *Oceanobacillus*. The set of bacteria that were found to be unique to the soil sample SU, includes *Anaerotruncus*, *Dialister*, *Rhodoferax*, *Parvimonas*, *Negativicoccus*, *Hoeflea*, and *Ruegeria*. The soil sample in SV condition, revealed a unique set of bacterial genera which include *Rhodoplanes*, *Neochlamydia*, *Byssovorax*, *Thermogutta*, *Verrucomicrobium*, *Luedemannella*, and *Tahibacter*. When exposed to the treatment with the defined bacterial consortium, the field soil sample in SBC condition exhibited a unique bacterial profile consisting of *Thauera*, *Ignavibacterium*, *Thermoactinomyces*, *Solitalea*, *Syntrophobacter*, *Fluviicola* and *Solimonas*. Under the concerted application of vermicompost and the bacterial consortium (SVBC), a unique bacterial profile was isolated from the experimental soil which included *Rhodanobacter*, GpV, *Clostridium*, *Okibacterium*, *Dokdonella*, *Phycicoccus*, and *Pedobacter*. The comparative Venn diagram of the common and unique bacterial members among the five samples under study, depicts that the enriched soil shows a higher number of unique members indicative of an improvement in overall soil health thus, indicating promotion of more and more associative microbial assemblage (Figure 9).

The functional genera in the field soil sample treated jointly with vermicompost and selected bacterial consortium in the presence of soybean plant (SVBC) showed the highest level of enrichment. However, important PGPB genera show an intermediate level of abundance in the case of the soil samples treated with bacterial consortia (SBC) and vermicompost (SV), independently. The untreated field soil (S) and soil sample SU exhibited the lowest level of abundance in the functional genera (Figure 10).

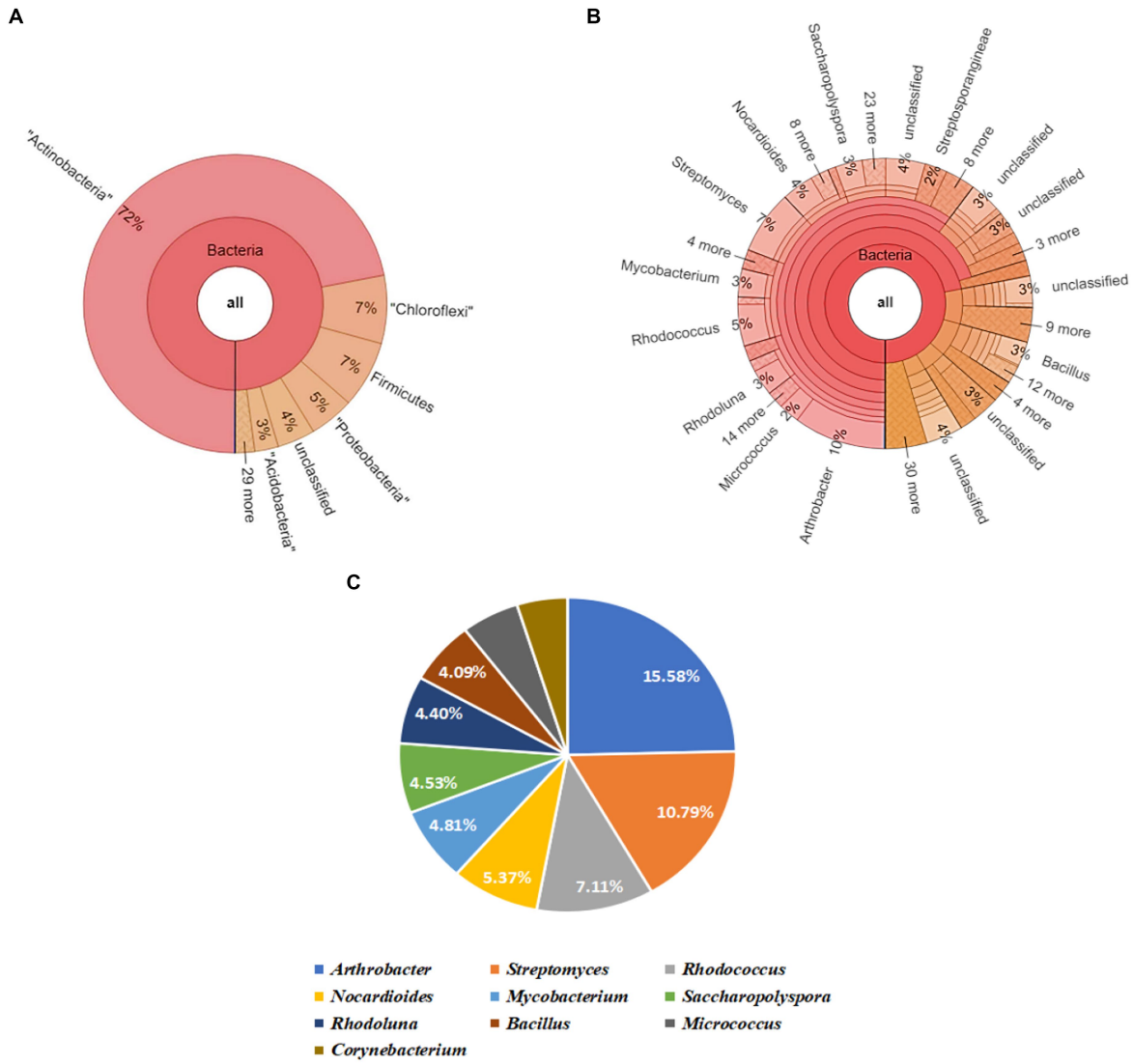

FIGURE 4
Bacterial abundance in S (untreated field soil) condition. **(A)** Krona chart representation of Phyla level abundance of prevalent bacterial assemblage; **(B)** Krona chart representation of Genera level abundance of prevalent bacterial assemblage; **(C)** Pie chart representing the top 10 scoring genera.

3.5 Functional profiling of the soil sample under varying experimental conditions

Further downstream analysis of the target genera like *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microbacterium*, *Paenibacillus*, *Bacillus*, and *Pseudomonas* reveals a stringent relationship in terms of the abundance of growth-promoting bacterial members which can be interpreted to be indicative markers across the soil samples under study. A large number of biological pathways, both homeotic and response, were predicted. The common metabolic pathways found to be of highest prevalence across the soil samples under differential experimental parameters under study were tryptophan metabolism and terpenoid backbone synthesis for soil S; starch and sucrose metabolism and quorum sensing in SU; valine, leucine, and isoleucine degradation for SV, and all the enriched metabolic cascades were found to be uniformly active in SBC or SBVC condition. Among the five datasets, SBVC exhibited a significantly higher magnitude of activation of the most prevalent functional pathways as compared to the remaining datasets in the presented heat map (Figure 11).

The intricate web of soil microbial interactions is pivotal in defining the ecological and functional attributes of an ecosystem. Within this matrix, the soil microbial network analysis of common genera provides a comprehensive insight into the dynamic relationships these microbes maintain. This network, which presents both uni-directional (where one microbe influences another without reciprocal action) and bi-directional (mutually beneficial or antagonistic interactions) connections, captures the interdependence and synergy of microbial populations. Delving deeper into this network, functional correlation provides a more nuanced understanding. In this layout, the nodes symbolize distinct microbial entities, while the connecting edges signify shared functional contributions. This means that two connected microbes are likely collaborating or competing in some capacity, potentially impacting specific metabolic pathways or ecological functions. For instance, two

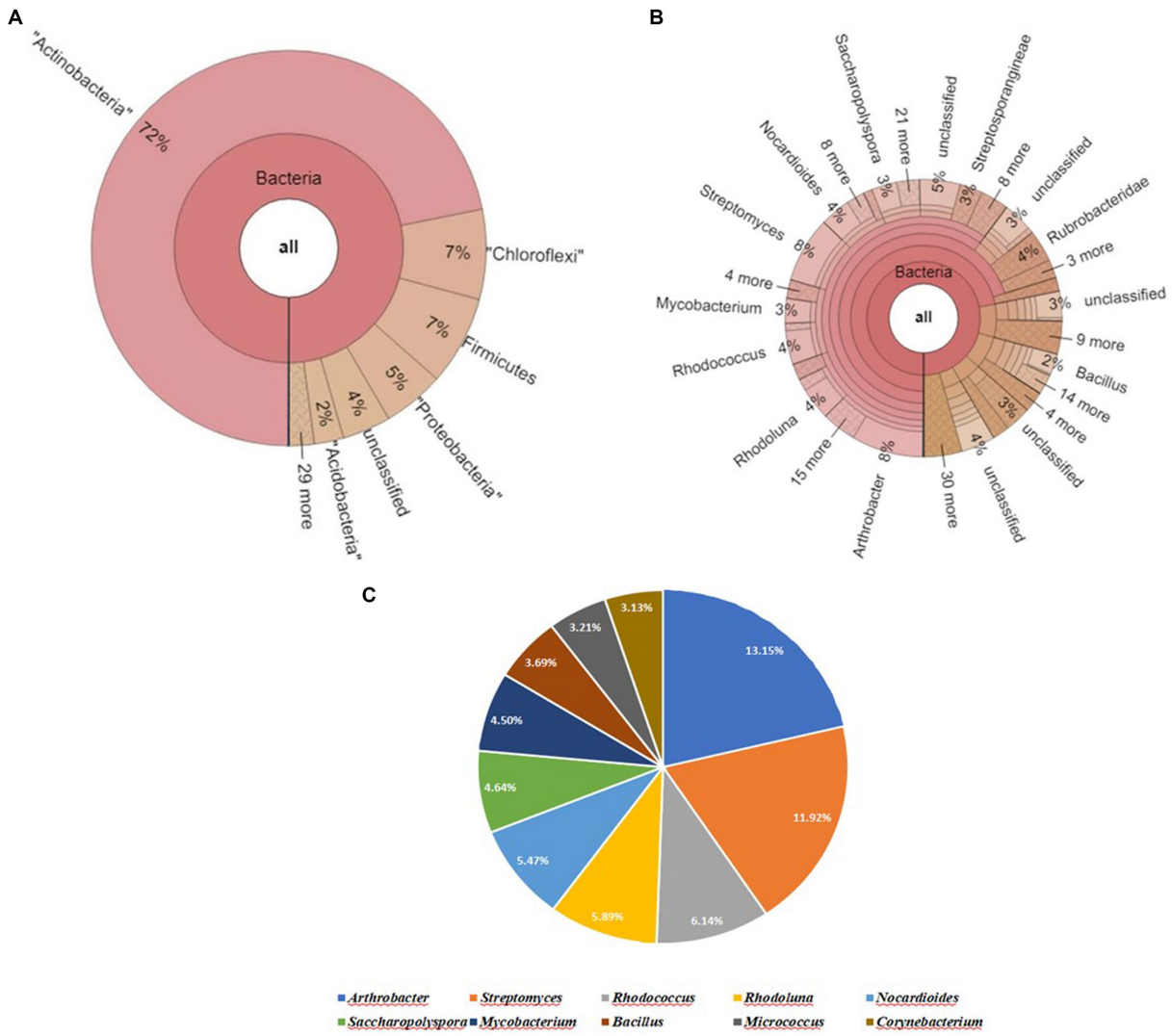

FIGURE 5
Bacterial abundance in SU (Untreated field soil with soybean plants) condition. **(A)** Krona chart representation of Phyla level abundance of prevalent bacterial assemblage, **(B)** Krona chart representation of Genera level abundance of prevalent bacterial assemblage, **(C)** Pie chart representing the top 10 scoring genera.

microbes might co-contribute to nitrogen fixation, or one might produce a substrate that the other utilizes. The referenced Figure 12 would likely present a visual representation of this network, highlighting the complexities and nuances of these interactions. Such networks not only help in understanding the current microbial dynamics but also in predicting how changes in one microbial population might impact others. This information is essential for soil health, plant growth, and broader ecosystem stability, especially in the context of environmental changes and sustainable agricultural practices.

4 Discussion

Microorganisms are, without a doubt, essential pillars of our ecosystem, demonstrating resilience by flourishing in a range of environmental conditions. Their versatility and adaptability have been highlighted by key international bodies such as the United Nations General Assembly (UNGA) Science Summit. Soil is the abode of myriad of microbial communities that encompass a bewildering array of physiological, metabolic, and genomic diversity essential for sustenance of soil fertility. Over-exploitation of arable lands with persistent application of agro-chemicals, inadequate return of organic matter to cultivated land, monoculture, and soil erosion, have been negatively impacting soil structural and functional properties resulting in depletion of nutrients, lowering in microbiological diversity, soil fertility, and crop productivity (Huang et al., 2019). Sustainability of agricultural systems has become a major challenge across the world as well as, in India where 54.6% of the total workforce depends on agricultural sector for their livelihood. Literature mining indicates that restoration of microbial diversity may help to recover such damaged agro-ecosystem replenishing various plant-beneficial services at community-level, consequently, improving plant health (Delgado-Baquerizo et al., 2016). Among

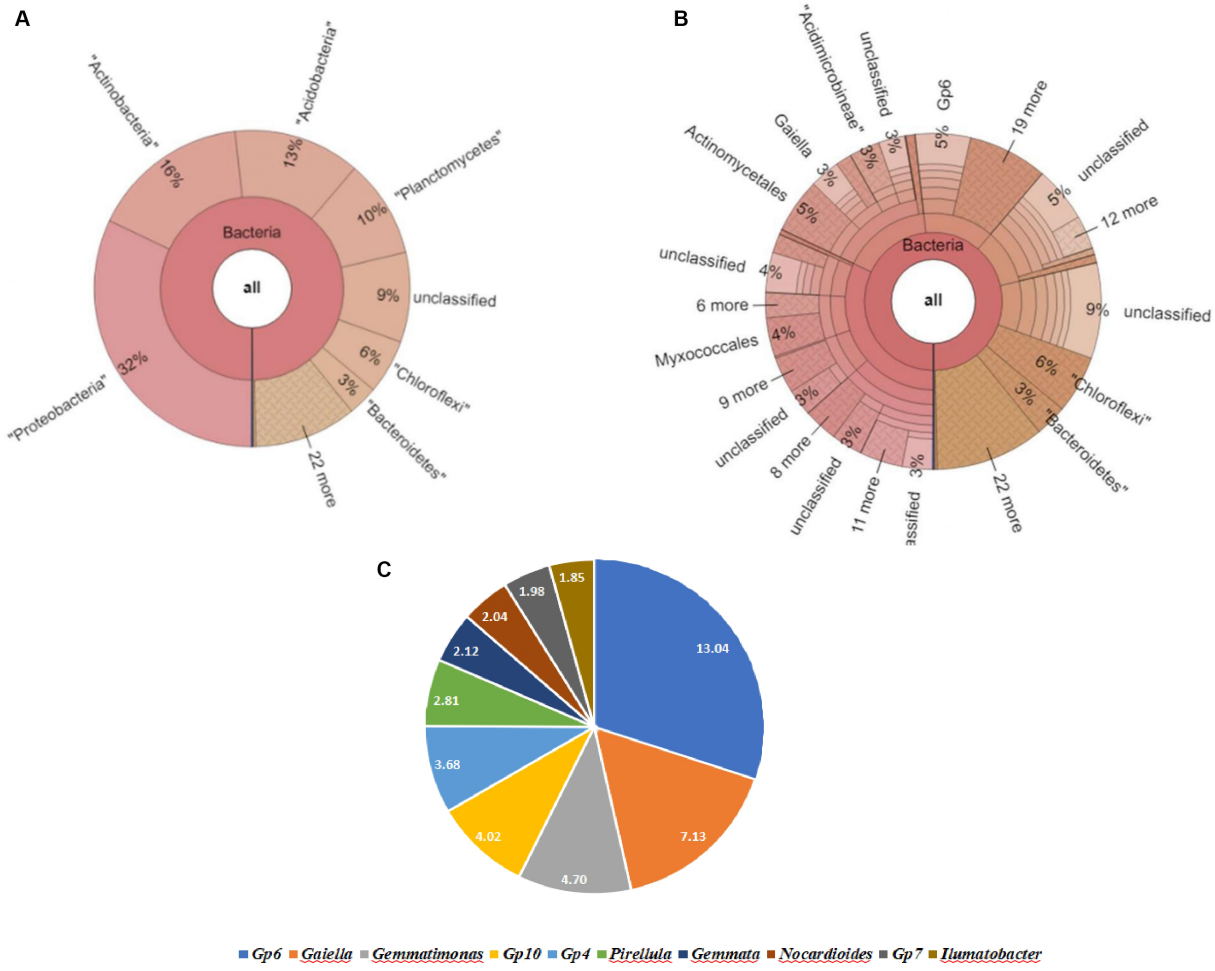

FIGURE 6
Bacterial abundance in SV (Field soil treated with vermicompost + soybeanplants) condition. **(A)** Krona chart representation of Phyla level abundance of prevalent bacterial assemblage, **(B)** Krona chart representation of Genera level abundance of prevalent. **(C)** Pie chart representing the top10scoring genera.

the BSMs, plant growth promoting bacteria (PGPB) can directly influence plant structure and performance through mobilization of soil nutrients, secretion of plant beneficial secondary metabolites (such as phytohormones, siderophore) as well as indirectly protect plants from biotic and abiotic stresses (Gouda et al., 2018). Furthermore, they are involved in maintaining physical, chemical and biological health of soil. Beneficial soil microbes are assumed to steer the bio-based revolution in the near future as a potential alternative to complement or replace chemical fertilizers and pesticides (Manfredini et al., 2021; Malusà et al., 2021). The introduction and promotion of Nature Based Solutions (NBS) echo one of the several facets of the UN SDGs to fight some of the world's greatest challenges like poverty and hunger (FAO, 2022). In the present investigation, three potent PGPB, *Bacillus subtilis* strain MMAM, *Bacillus zhanzhouensis* strain MMAM, and *Bacillus cereus* strain MMAM, isolated from an over-used arable land, were used as a composite bio-inoculant for plant-growth promotion study in the soil from the same nutrient-depleted field. These three bacterial isolates possessed multifarious PGP activity such as mineral nutrient (NPK) harnessing ability, production of IAA, GA, ACC deaminase, siderophore, and biofilm, several lytic enzymes, HCN, and NH_3 in *in vitro* conditions (Mukhopadhyay, 2022). The effects of the inoculum on the growth promotion of soybean plants in vermicompost-treated and untreated field soil were evaluated. Furthermore, the implications of the amendments on the resident soil bacterial community were analyzed. As per the pot trial experiment, a significant improvement with respect to vegetative and reproductive parameters of the test plant were observed in varying degrees in amended conditions. The highest improvement was recorded in SBVC followed by SBC and SV set-up. Earlier researchers showed that improvement in crop yield can be achieved through indigenous or inoculated PGPB via enhanced nutrient availability or phytohormones production (Becker et al., 2018; Bechtaoui et al., 2020). The *de novo* biosynthesis of plant growth enhancers (such as cytokinins and IAA) synergistically reinforces the phytohormone signaling cascades thereby, augmenting host tolerance to various biotic and abiotic stresses from the environment that they are constantly subjected to Naveed et al. (2015). Many soil bacteria are able to produce a plethora of hydrolytic enzymes, which are directly associated with the mineralization of organic materials thus, facilitating the nutrient mineralization and carbon cycling process

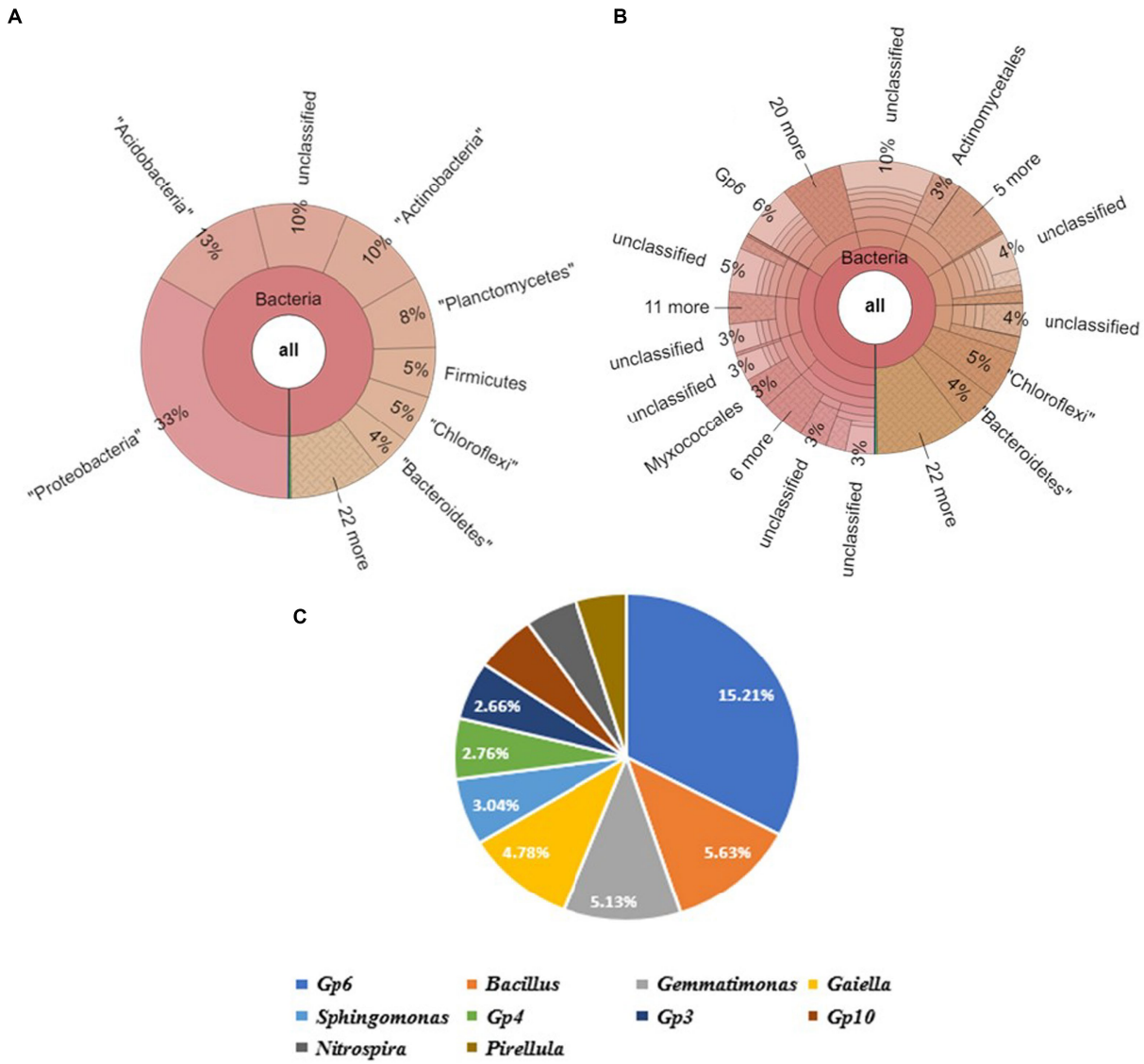

FIGURE 7
Bacterial abundance in SBC (Field soil treated bacterial consortium+soybean plant) condition. **(A)** Krona chart representation of Phyla level abundance of prevalent bacterial assemblage, **(B)** Krona chart representation of Genera level abundance of prevalent bacterial assemblage, **(C)** Pie chart representing the top 10 scoring genera.

(Sinsabaugh et al., 2008). Positive impacts on plant growth due to *Bacillus*-induced enhanced nutrient acquisition and hormonal modulations following treatment with *Bacillus*-based formulations have been observed in recent studies (Tsotetsi et al., 2022). Furthermore, Hu et al. (2021) reported that application of multi-strain microbial consortia inoculants (*Pseudomonas* spp.) is capable of enhancing plant growth more effectively compared to that of single-strain inoculants. Co-inoculation of *Glycine max* L. plants with *Bradyrhizobium japonicum* and *Azospirillum brasilense* inoculants showed outstanding results for improving grain yield and nodulation over that of the non-inoculated control (Hungria et al., 2013). A recent study reported that a composite inoculum of *Pseudomonas chlororaphis* H1 and *Bacillus altitudinis* Y1 could remarkably enhance soybean plant growth, yield performance, enrich the beneficial bacterial composition around root and rhizospheric region with a positive effect on soil improvement (Zhang et al., 2023). Thus, our findings are in line with the previous studies in this arena.

According to Vassileva et al. (2020), the bio-inocula of multi-strain microorganisms with different plant-beneficial properties may exert a consistent impact in on plant productivity in field conditions, either due to the complementation effects of plant-favorable functions at the consortium level or because of imminent diversity effects in the plant-associated microbiome (Hassani et al., 2018). In the present work, enhanced vigor and performance of soybean plant, might have occurred in the consortium-treated condition, due to the composite PGP mechanisms exerted by the three potent PGPB isolates, *Bacillus subtilis* strain MMAM, *Bacillus zhanzhouensis* strain MMAM, and *Bacillus cereus* strain MMAM. Additionally, this study indicated that soil amendment with vermicompost might have an added advantage

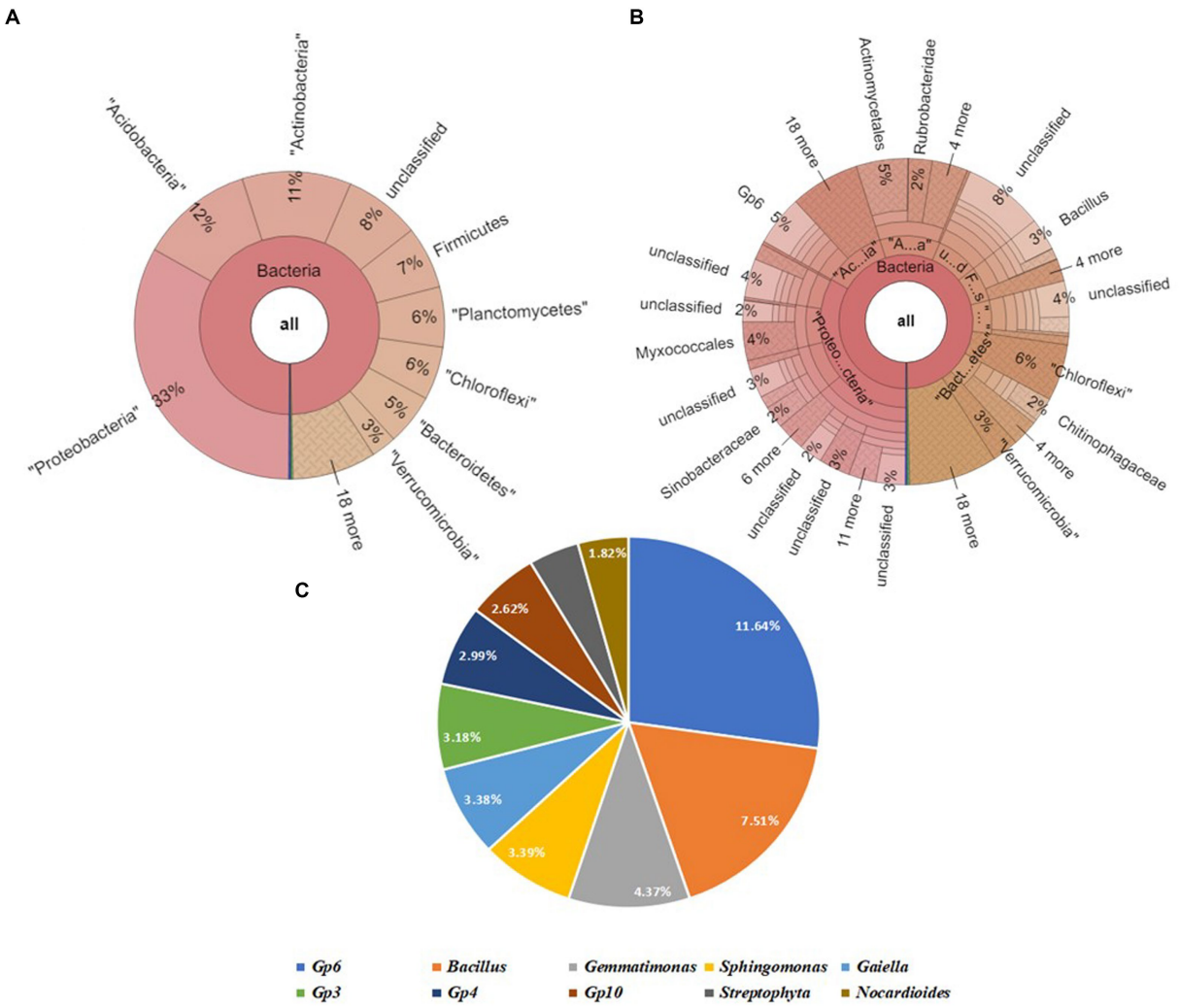

FIGURE 8
Bacterial abundance in SVBC (Field soil treated with vermicompost +bacterial consortium + soybean plants) condition. **(A)** Krona chart representation of Phyla level abundance of prevalent bacterial assemblage, **(B)** Krona chart representation of Genera level abundance of prevalent bacterial assemblage, **(C)** Pie chart representing the top 10 scoring genera.

for plant growth promotion, both in consortium-treated and untreated soil conditions over to that of the only bacterial inoculant treated condition. Vermicompost probably acted as a soil prebiotic to increase the population of resident associative beneficial bacteria and also as a nutrient source for the bacterial strains already existing within the soil–plant system (Arancon et al., 2006; Strachel et al., 2017; Vassileva et al., 2020).

In the recent years, the implications of introduced bioinoculants on soil microbial community composition are extensively investigated. Xing et al. (2022) explored the effect of co-inoculation with three beneficial bacteria (*Bradyrhizobium japonicum* 5,038 (R5038), *Bacillus aryabhattai* MB35-5 (BA) and *Paenibacillus mucilaginosus* 3,016 PM), alone and in combination, on soybean rhizosphere bacterial community composition and on the soil properties. Their findings confirms that several PGPB with multifaceted functions could effectively be used together as composite bacterial inoculants, which coordinately shift the rhizospheric bacterial community structure and improve plant performance. In our study, the analysis of metagenomic data sets of treated and untreated soil, indicated a modulation of soil bacterial community composition following soil augmentation. According to Willis Rarefaction (2019), analysis of the alpha diversity in amplicon sequencing data appears to be a common first approach to measuring differences between environments in terms of microbial ecology to summarize an ecological community structure according to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups) or both. In the present work, the diversity profiles reveals that although the 5th dataset (SVBC) i.e., field soil treated with both vermicompost and bacterial consortia in the presence of soybean plant, had a higher index of Shannon diversity, thus, establishing a higher richness and uniformity in distribution of the total number of genera in the given sample. Our findings are in line with Ansari et al. (2023) who observed a positive correlation between the diversity of soil microbiota and availability of soil nutrients such as, organic carbon, available N and K content, thereby influencing plant growth.

The set of metagenomic analyzes carried out demonstrated that Actinobacteria was found to be the most prevalent phylum in the

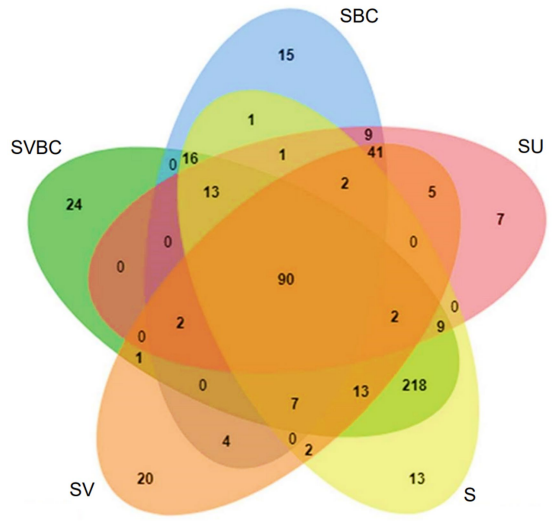

FIGURE 9
Comparative Venn diagram depicting the common and unique bacterial members among the five samples under study.

first two datasets, i.e., untreated field soil and that which was treated with vermicompost in the presence of soybean plant. However, a pronounced shift of phyla was observed in all the amended experimental datasets toward Proteobacteria. This change in abundance could be possibly indicative of the fact that the beneficial Proteobacteria have been shown to exhibit multifaceted roles contributing to plant growth and development such as promoting nutrient balance and acquisition via nitrogen fixation (Mirza et al., 2001; Miliute et al., 2015). The sustained presence of Actinobacteria as one of the common abundant phyla suggests their involvement in nutrient cycling, improvement in soil quality, and enhancing crop yield along with maintenance of plant health thus, being a reliable contender as a biofertilizer alternative to conventional inorganic supplements in agricultural (Boubekri et al., 2022). Various levels of ubiquity were explored as well, regarding the diversity profiles of the bacterial genera across all the datasets including common and unique genera. Some of the rhizobial and PGP bacterial assemblages found to be common among all the treated datasets were *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microbacterium*, *Paenibacillus*, *Bacillus*, and *Pseudomonas*, as mentioned in the results section. In soybean plants, acquisition of

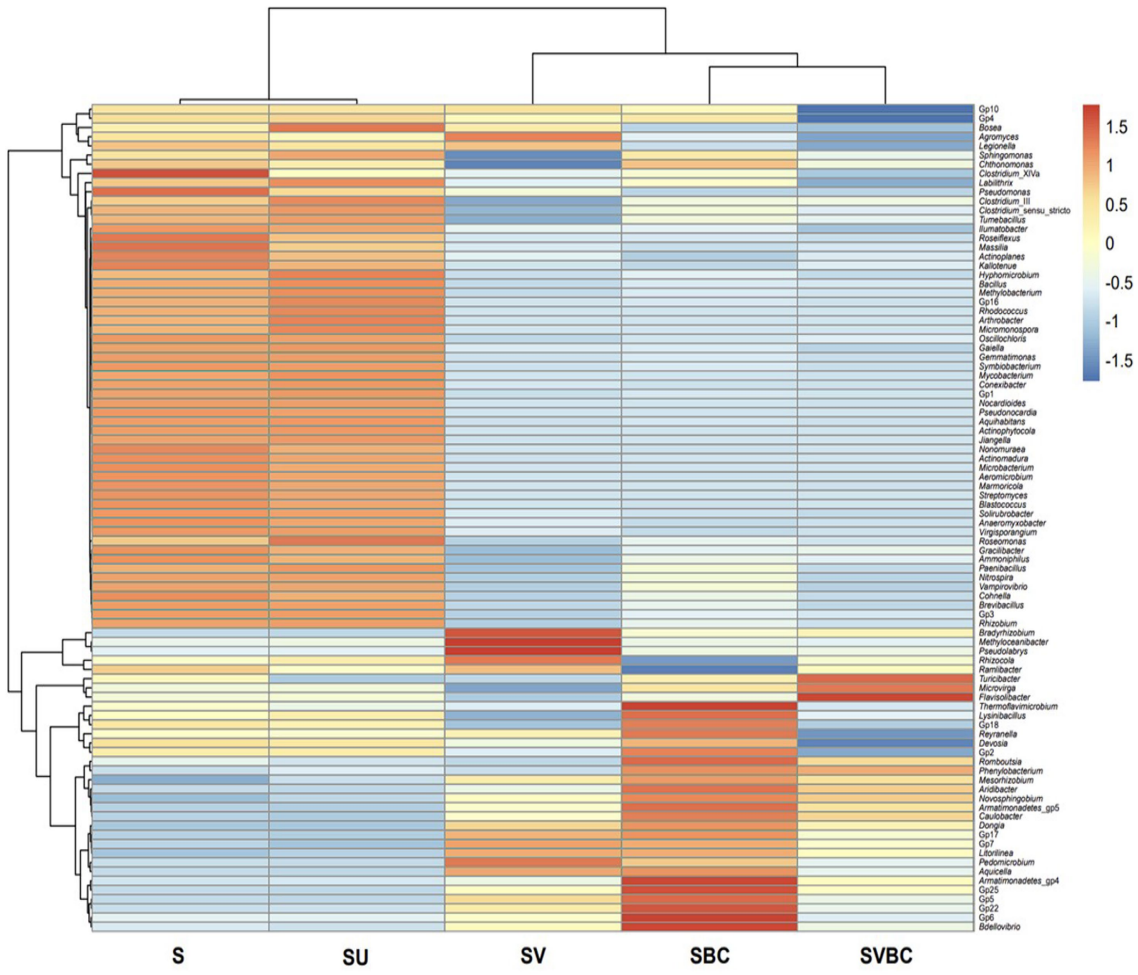

FIGURE 10
Heatmap depicting the abundance of important growth-promoting bacterial members in the five samples under study.

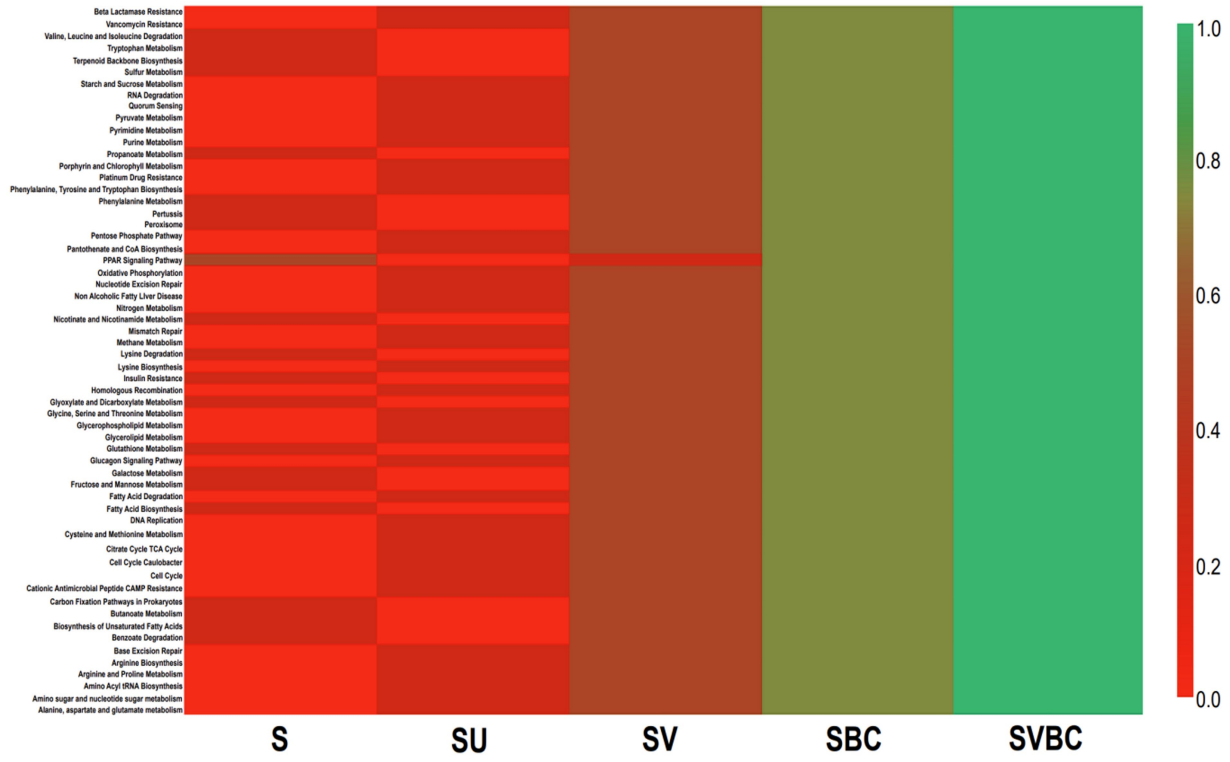

FIGURE 11
Heat map of functional profiling showing variations in the predicted active pathways of the common genera in the field soil obtained from controlled and experimental conditions.

phosphorus has been specifically evidenced whereby, the inorganic phosphate remobilization via *Rhizobium* is postulated to be mechanistically driven by rhizospheric acidification which is enhanced in the case of modulation between the plant-microbe holobiome (Qin et al., 2011). Furthermore, rhizobial strains have been shown to secrete and/or produce 1-aminocyclopropane 1-carboxylate (ACC) deaminase, siderophores, and extracellular polysaccharide for combating osmotic and heavy metal stresses thus, cumulatively boosting soybean seed germination under drought conditions (Igiehon et al., 2019). Advantages of inoculation of soybean plants with selected strains of *Bradyrhizobium japonicum* exposed to salt stress in greenhouse conditions have been reported to restrict mineral nutrient uptake along with amplified antioxidant activity and production of glutathione reductase, ascorbate peroxidase, and malondialdehyde along with other protective osmolytes thereby, ameliorating the hypersaline microenvironment which otherwise limits the nodulation potential, yield, plant growth and rate of photosynthesis in soybean plants (Han and Lee, 2005). Agricultural sustainability stems majorly from biological nitrogen fixation (BNF), 45% of which is exploited in current agricultural practices. Furthermore, 80% of BNF are contributed by leguminous plant-microbe associations between *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* and their abundances are dictated by ecological, edaphic, genetic and agronomic parameters (Sindhu et al., 2019). Literature sources also reveal that selected strains of *Pseudomonas* have been identified which can substantiate the productivity of the soybean-wheat cropping system in regions of central India with an enhanced content of clayey minerals in the soil, whereby they were found to be boosting soil enzyme activities, total system productivity and nutrient uptake in field trial (Sharma, 2011). Moreover, most pseudomonads have been found to produce phytohormones like IAA along with secondary metabolites including antibiotics with antifungal activity. An interesting study investigated the synergistic effect of inoculating selected strains of *Pseudomonas aeruginosa* with *Bradyrhizobium japonicum* for their potential implication as a biofertilizer consortium for soybean. Compatible strains revealed elevated solubilization of inorganic phosphate and production of IAA, ACC deaminase, and biofilm biosynthesis along with improved grain yield, symbiotic and soil quality parameters compared to independent inoculation with single strains (Kumawat et al., 2019). A specific strain of *Bacillus aryabhattai* has been evidenced to significantly improve the growth of soybean via the synthesis of substantial amounts of abscissic acid, IAA, cytokinins, and GA with subsequent induction of heat stress tolerance (Park et al., 2017). The aerobic endospore-forming bacteria belonging to the genera of *Bacillus* and *Paenibacillus* have been reportedly involved in atmospheric nitrogen fixation, phosphate solubilization, biofilm formation, and production of microbicidal metabolites. They have been evidenced to be mobilizing host plant nutrition thereby supporting their growth, along with antagonizing pathogenic infestations of insect pests, bacteria, fungi, and nematodes by modulation of host defense cascades and triggering induced systemic resistance (ISR) thus making them suitable contenders for application in sustainable agricultural practices (Govindasamy et al., 2010).

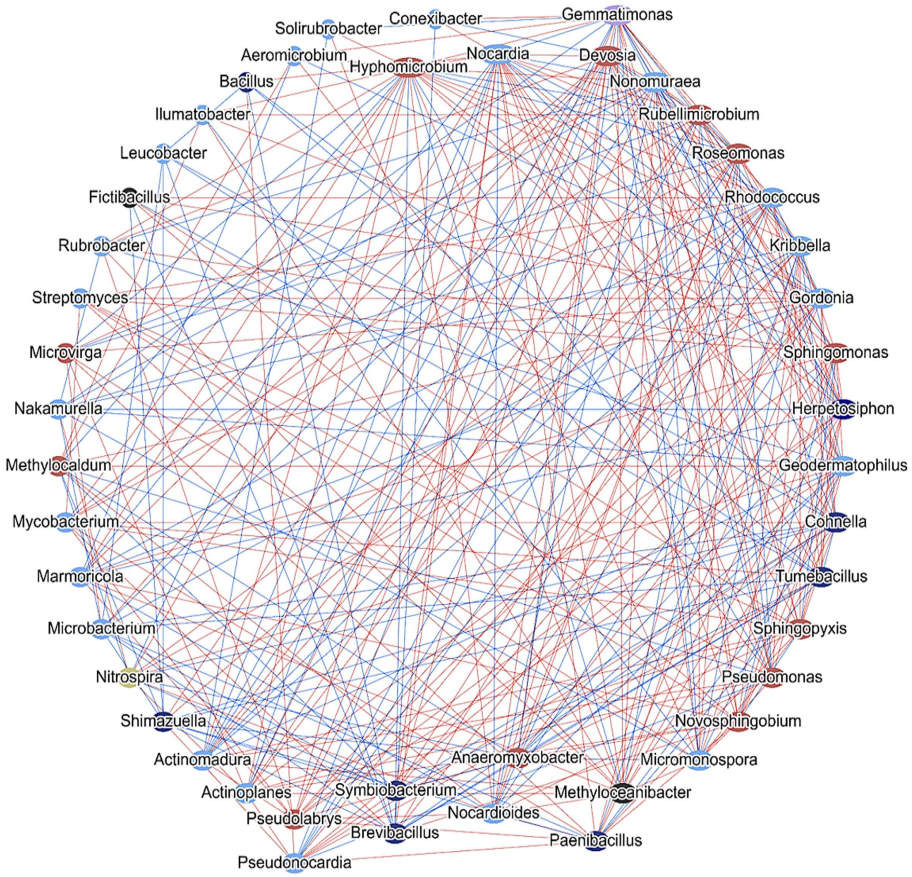

FIGURE 12
Network analysis showing cross-talk between different genera in the field soil consortia obtained from controlled and experimental conditions. Blue lines and red lines indicate bidirectional and unidirectional interactions.

During the investigation, a large number of biological pathways were also predicted, which encompassed both homeotic and response pathways. The common metabolic pathways that were found to be of highest prevalence across the soil samples under differential experimental parameters under study were tryptophan metabolism and terpenoid backbone synthesis for the untreated field soil; starch and sucrose metabolism and quorum sensing for field soil in presence of soybean plant; valine, leucine and isoleucine degradation for field soil treated with vermicompost in presence of soybean plant, and all the enriched metabolic cascades were found to be uniformly active in cases of field soil treated with a selected bacterial consortium or with both the consortia and vermicompost in presence of soybean plant. Among the five datasets, the combined treatment of vermicompost with the selected bacterial consortium exhibited a significantly higher magnitude of activation of the most prevalent functional pathways as compared to the remaining datasets in the presented heat map. The selectively enriched pathways of terpenoid backbone synthesis in almost all the datasets can be correlated with existing literature sources which substantiate this functionality in *Salvia miltiorrhiza* seeds from seven different geographic origins whereby, it has shown to provide important precursors for terpenoid biosynthesis thus, indicating a significant level of secondary metabolism for enhancing biotic and abiotic stress resistance (Chen et al., 2018). A significant down-regulation of the sucrose and starch metabolism pathways can be noted for the untreated field soil in the presence of the soybean plant which might be suggestive of the adaptive trait of specialized and dynamic carbon utilization from sources like α-pinene, naphthalene secreted in the root exudates as a part of the unique microenvironment utilized by bacteria like *Pseudomonas*, *Burkholderia*, *Mycobacterium*, *Streptomyces*, *Sphingomonas*, *Pseudomonas*, *Ralstonia*, etc. in the rhizospheric bacterial consortium in soybean thus, leading to a decrease in common carbon metabolism pathways (Liu et al., 2019). Glutathione up-regulation was seen to be considerably activated in most of the soil samples which in synergy with functionalities like geraniol disintegration, limonene, naphthalene, and pinene degradation have potential implications in bioremediation of xenobiotic contamination (Liu et al., 2019).

The soil microbial network analysis of common genera between the 5 experimental conditions exhibits a complex network that represents the interactions that take place between the field soil bacterial assemblages. Here, we found two patterns of interactions, one in which both microbes appear to be communicating with each other. The bidirectional phenomenon is represented by blue lines comprising of members such as *Pseudomonas*, *Solirubrobacter*, *Phenylobacterium*, etc. The second pattern is unidirectional contact which is being mediated by any one member toward the other. This is

represented by red lines, exhibited by *Streptomyces*, *Rhodococcus*, *Mycobacterium*, *Paenibacillus*, etc. The edges in between the nodes of the network indicating that some genera such as, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Nitrospira* are (potentially) co-contributing to one or more specific functions. These findings are supported by the report of Ma et al. (2018), where a significant over-representation of several bacterial classes and genera were observed to be involved in symbiotic N-fixation, plant health promotion, biol-control and soil catalase activity promotion, following bacterial inoculation treatment. Furthermore, a decrease in some taxa with negative impacts on soil quality, was noticed in this study (Ma et al., 2018). The analysis of soil bacterial community also revealed that, application of the microbial consortium resulted in an elevated crosstalk among the microbial members of the niche. Along with an increase in crosstalk, elevated expression of metabolic pathways was also observed, indicating a modulation of resident bacterial assemblage at the community level toward the improvement in soil biological health.

Finally, the results of the current study indicated that the application of the composite inoculant of residual PGPB strains to the soil, in combination with vermicompost, might have enriched the agriculturally beneficial soil microbial assemblages already present in the microbiome and the resultant effects have been reflected in plant growth promotion. This unique soil augmentation strategy has multi-pronged beneficial aspects. Implementation of this technology can effectively enhance vegetative and reproductive performance of plants targeting to increase agricultural productivity. Post-amendment increased abundance of plant beneficial microbial assemblages resulted an enrichment of soil microbial flora leading to an improvement in soil biological health. The rejuvenated patched of over-used land can be used by the small and marginal farmers for cultivation of resilient, as well as profitable crops. In the long run, it can lead to a societal benefit to improve the economic status of the poor farmers. In ecological aspect, popularization of this technology will promote sustainable agriculture. The limitation of the present work is evaluation of the novel strategy at pot trial condition. Extensive field trials are needed to ascertain its implications at field condition usually, some laboratory-tested products fail to exert promising results under field trial conditions. Soybean was used as a model crop in our study, various other cropping systems should also be explored to validate the growth enhancing and stress-relieving behavior of the tested PGPR consortium. Major challenges of this study are: availability of the formulation to the farmers; generation of public awareness and arrangement of proper training facility at the rural perspective for popularizing this novel technology.

5 Conclusion

As the world grapples with burgeoning populations and the concomitant challenges of ensuring food security, microbial innovations could be the linchpin. The current work reported that the novel multi-strain inoculant three native Bacillus spp. could remarkably enhance soybean plant growth, yield performance and simultaneously, enrich the resident functional bacterial assemblage in soil. Furthermore, the upgraded reclaimed soil can be successfully used for growing the 'golden bean', soybean which is still under-utilized in the state of West Bengal, India, thus helping to improve socio-economic status of the marginal and landless farmers of this region. There is still a scarcity of microbial inoculants-based good products in the market. In this context, the utilization of native BSM to enhance plant productivity in over-exploited nutrient-depleted arable soil through modulation resident microbiome, can emerge as a promising strategy for futuristic agriculture transforming barren patches to fertile expanses, and reducing the environmental footprint of traditional cultivation practices. By harnessing "Emerging Trends and Advances in the Socioe-conomic Applications of Beneficial Microbes," we are not just looking at scientific advancements but a redefinition of socio-economic paradigms.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

MM: Data curation, Investigation, Writing – original draft, Conceptualization, Formal analysis, Methodology, Resources, Software, Validation, Writing – review & editing. AM: Formal analysis, Methodology, Software, Validation, Writing – review & editing. SG: Methodology, Methodology, Validation, Writing – review & editing, Data curation. AC: Methodology, Software, Formal analysis, Investigation, Writing – review & editing. SR: Formal analysis, Methodology, Software, Writing – review & editing. SC: Supervision, Validation, Writing – review & editing. VS: Validation, Funding acquisition, Project administration, Resources, Writing – review & editing. VK: Funding acquisition, Project administration, Resources, Validation, Writing – review & editing. AS: Formal analysis, Methodology, Software, Writing – review & editing. FE-D: Formal analysis, Funding acquisition, Methodology, Software, Writing – review & editing. MA: Formal analysis, Funding acquisition, Resources, Software, Writing – review & editing. AŞ: Formal analysis, Resources, Software, Validation, Writing – review & editing. BD: Data curation, Investigation, Project administration, Writing – review & editing. AKM: Conceptualization, Investigation, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Unveiling *Curvularia tuberculata*-induced leaf anomalies in *Rhododendron ferrugineum*: implications in cultural-ecological conservation and harnessing microbial intervention in socio-economic advancement

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Introduction: The research focuses on *Rhododendron ferrugineum* L., Nepal's national flower and Uttarakhand's state tree, thriving in high-altitude mountain ecosystems.

Methodology and Result: A study conducted in Himachal Pradesh (Latitude: N 31° 6′ 2.0088″, Longitude: E 77° 10′ 29.9136″) identified leaf anomalies resembling rust-like manifestations in *R. ferrugineum*. These anomalies were traced back to the pathogenic fungus *Curvularia tuberculata*, marking the first documented case of its impact on *R. ferrugineum* in India.

Discussion: This discovery emphasizes the need for vigilant monitoring, disease management research, and conservation efforts to protect the cultural and ecological significance of this iconic shrub. Beyond its immediate findings, the study introduces a novel dimension to Indian flora by associating *C. tuberculata* with *R. ferrugineum*, historically linked to monocotyledonous crops. The research methodology combines traditional microscopic examination with

advanced genomic sequencing and phylogenetic analysis, enhancing pathogen identification accuracy.

Future prospect: In a broader context, this research aligns with the United Nations Sustainable Development Goals (SDGs) by highlighting the importance of environmental preservation, conservation, and sustainable management. It underscores the intricate interplay between biodiversity, cultural heritage, and the need for holistic solutions. Overall, this study calls for proactive measures to protect *R. ferrugineum's* cultural and ecological heritage and emphasizes the significance of interdisciplinary approaches in addressing emerging ecological threats.

KEYWORDS

plant pathology, biotherapeutics, microbe-assisted bioremediation, synthetic biology, leaf infection, *R. ferrugineum*, *C. tuberculata*, cold-adapted pathogen

1 Introduction

Global ecosystems, encompassing diverse landscapes from agricultural fields to natural floral ecosystems, are confronted with an ever-evolving and intricate challenge posed by pathogenic fungi. This challenge is particularly pronounced in India, a country renowned for its ecological diversity characterized by a wide range of climatic zones and an abundance of native flora. Within this context, India has become a pivotal focal point for the observation and study of these complex ecological interactions. One of the pivotal factors amplifying the impact of pathogenic fungi on India's ecosystems is the changing environmental conditions. Specifically, the escalation of temperatures and the intensification of heatwaves have significantly heightened the vulnerability of India's native flora. Regions such as the Himalayan foothills, with their unique ecosystems, and the biodiverse expanse of the Western Ghats, are experiencing a surge in disturbances that not only disrupt the delicate ecological balance but also wield substantial economic consequences for regional agriculture and associated industries (Chaloner et al., 2021). An alarming and noteworthy facet of this narrative is the emergence and adaptability of trans-kingdom fungi, a phenomenon that transcends conventional taxonomic boundaries. These pathogens, primarily originating from the *Ascomycota* and *Glomeromycota* phyla (Gauthier and Keller, 2013), possess an extraordinary capability to infect hosts spanning multiple kingdoms. This remarkable adaptability creates a scenario where plant species once considered resistant are now under the looming threat of fungal infections. Moreover, some of these fungi have displayed the disconcerting tendency to affect human hosts, thereby introducing a new dimension to this ecological challenge and raising significant concerns for public health. To delve even further into the complexity of this issue, it is crucial to recognize the multifaceted nature of the interactions between pathogenic fungi and their hosts. The mechanisms that underlie fungal adaptability and host susceptibility are intricate and multifarious, encompassing genetic, physiological, and environmental factors. Consequently, the urgent imperative lies in conducting comprehensive research, proactive conservation efforts, and innovative strategies to mitigate the multifaceted impact of pathogenic fungi on ecosystems, agriculture, and human well-being. In conclusion, the challenges posed by pathogenic fungi in global ecosystems, with a specific emphasis on India's unique ecological diversity, demand in-depth exploration and comprehensive understanding. This includes elucidating the mechanisms of fungal adaptability, identifying host susceptibility factors, and devising innovative solutions to safeguard not only India's rich biodiversity but also its agricultural sustainability and public health.

The mechanics underlying these fungal incursions are both fascinating and concerning. From an ecological standpoint, the dispersal methods of these fungi—via wind, water, or animal vectors—are critical (Epstein and Nicholson, 2006). The initial adherence of spores to potential host surfaces, driven by specialized adhesive molecules, often heralds the onset of an infection cycle. This subsequently evolves into a complex interplay of germination, penetration, and establishment within the host. Two fungal species stand out in this emerging narrative: *Exserohilum rostratum* and *C. tuberculata* (Figure 1). While their natural behavior as soil saprophytes (Doehlemann et al., 2017) has been documented, it's their newly observed pathogenic tendencies, especially towards an array of Indian plant species, that demand attention. Plants located close to human settlements are becoming inadvertent vectors for these crossover pathogens, complicating both ecological balance and public health considerations (Rinaldi et al., 1987). Our research has made a significant and pioneering contribution to the field of ecology and plant-pathogen dynamics, with a recent breakthrough discovery in the state of Himachal Pradesh, India. In this region, we have identified a novel host-pathogen interaction that has far-reaching implications. Specifically, the pathogen *C. tuberculata*, which has traditionally been associated with staple crops, is now encroaching upon the habitat of the iconic *R. ferrugineum*. However, its importance extends beyond ecological considerations; it serves as a symbol of cultural pride and represents a substantial portion of global biodiversity. Under the Rhododendron genus, there are a staggering 800 to 1,100 species (Bursill and Rouse, 1998), showcasing the diversity and richness of this plant family. Furthermore, the significance of *R. ferrugineum* transcends national borders, as it is not only recognized as the national flower

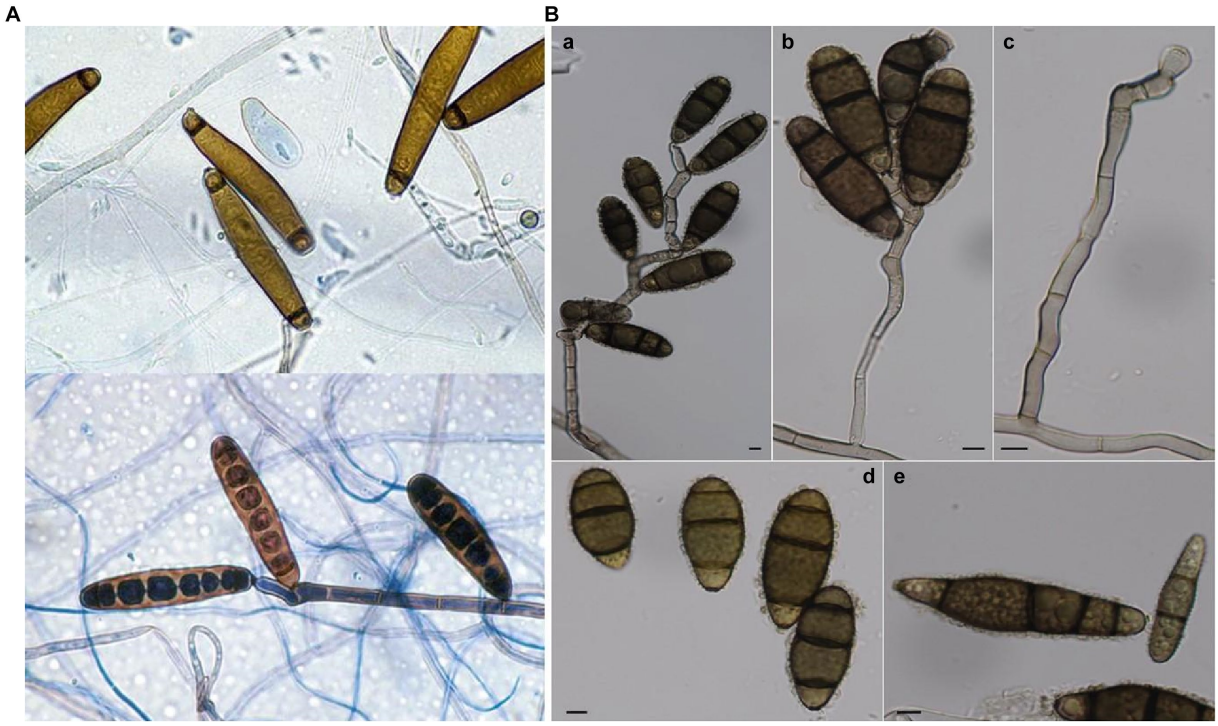

FIGURE 1
(A) Conidia of *E. rostratum* & Sympodially elongating conidiophore and conidia of *E. rostratum* [taken from open access source, ref.: (Domsch et al., 1980; Haq et al., 2020)]; **(B)** *C. tuberculata*: **(A–C)** Conidiophores and conidia. **(D,E)** Conidia (Bars = 5 μm, unless otherwise specified). [taken from open access source, ref.: (Jain, 1962; Zhang and Watson, 1997)].

of Nepal but also stands as an emblem of Himachal Pradesh's natural heritage (Kumar et al., 2019; Namgay and Sridith, 2020).

The colloquial name for *R. ferrugineum*, the 'Alpine rose,' evokes the image of a hardy and resilient plant that has thrived in challenging mountainous environments. Beyond its ecological and cultural importance, this species has a rich history intertwined with human traditions and traditional medicine practices (Popescu and Kopp, 2013). Historically, various parts of *R. ferrugineum* have been employed in the treatment of a wide array of ailments, ranging from arthritis to rheumatism (Hubert et al., 2022). However, the utilization of this plant for medicinal purposes also underscores the need for a careful and balanced approach. Its potential for adverse reactions (Louis et al., 2010) necessitates a nuanced understanding of its properties and the responsible use of its medicinal attributes.

Delving into the phytopathogenic tactics, these fungi exhibit a broad arsenal from enzyme-driven cellular breaches to specialized toxin production, all strategically orchestrated to compromise host defenses (Meng et al., 2009). *Curvularia* sp., as filamentous fungi with wide distribution and ecological significance, were the focus of our evolutionary analysis. We delved into the ITS sequences of various *Curvularia* isolates to unravel their evolutionary relationships. Additionally, we integrated bootstrap values to assess the reliability of the inferred phylogenetic relationships. The urgency of our study is further underscored by the previously documented predilection of *C. tuberculata* for North Indian crops (Majeed et al., 2016). In conclusion, our research has unveiled a fascinating and intricate host-pathogen interaction in Himachal Pradesh, shedding light on the evolving dynamics between *C. tuberculata* and *R. ferrugineum*. Beyond the ecological implications, this discovery highlights the cultural, biodiversity, and traditional medicinal significance of *R. ferrugineum*, emphasizing the need for both conservation efforts and responsible utilization of this invaluable resource. This newfound knowledge opens avenues for further research and underscores the interconnectedness of ecological, cultural, and medicinal aspects of plant species like *R. ferrugineum* in our natural world. By synthesizing historical data with contemporary findings, this research seeks to provide a holistic perspective on a novel fungal invasion, offering insights into its mechanisms, ecological ramifications, and potential countermeasures.

2 Materials and methods

2.1 Isolation and cultivation of pathogens from *Rhododendron ferrugineum* leaves

2.1.1 Sample procurement and preservation

Leaves of *R. ferrugineum* exhibiting symptomatic infections were systematically gathered during our recent expedition to Shimla, Himachal Pradesh (refer to Figure 2 for visual representation). To preserve the physiological and pathological state of these specimens, each was immediately kept in specialized hermetic bio-storage bags and refrigerated at a precise 4°C.

FIGURE 2
R. ferrugineum plants from where the infected leaves were collected, at summer hill, Shimla, Himachal Pradesh, India, and pathogenic protein of *C. tuberculata* induced Leaf Anomalies in *R. ferrugineum*.

2.1.2 Sample preparation and segmentation

Under sterile laminar flow conditions, targeted lesions indicative of pathogenic activity was delineated. Using sterilized surgical-grade instruments, these marked sections were excised into consistent, defined segments onto new sterile plates.

2.1.3 Comprehensive surface sterilization

A regimented decontamination process was instituted to obviate exogenous microbial presence. Commencing with an initial rinse in autoclaved distilled water, segments were then briefly exposed to a 75% ethanol solution. Subsequently, a 0.1% (w/v) mercuric chloride ($HgCl_2$) immersion ensured a deeper level of sterilization for precisely 1 min. This rigorous sterilization process culminated in a series of three exhaustive washes using sterile distilled water, after which any residual moisture was assiduously removed using autoclaved blotting materials.

2.1.4 Strategic inoculation procedure

Sterilized leaf fragments were gently placed onto specialized Potato Dextrose Agar (PDA) culture plates. To negate bacterial proliferation and maintain a fungus-specific culture environment, Streptomycin sulfate (HIMEDIA's premium batch TC035-5G) was

introduced at an optimized concentration of 100 ppm. This addition takes place before the agar solidifies after sterilization, since the antibiotic becomes inactive at temperatures higher than 55°C.

2.1.5 Monitored incubation

In our state-of-the-art incubation chamber, calibrated to a stable 30°C, the inoculated plates were maintained, fostering a conducive milieu for microbial propagation over an uninterrupted span of 48 h.

2.1.6 Sub-culturing for enhanced growth analysis

Following the initial incubation phase, prominent fungal colonies were selected for sub-culturing. This step involved the transfer of viable growth sections onto fresh PDA plates, each with the aforementioned Streptomycin concentration. The ensuing incubation was monitored and maintained at 30°C until optima fungal sporulation was observed.

2.1.7 Preservation for subsequent analyses

Optimal sporulation was determined through observation and quantification of matured cultures under controlled laboratory conditions. Specifically, sporulation was deemed optimal when a substantial number of mature, well-formed spores were visibly present within the culture. This evaluation included microscopic examination, scrutinizing the abundance, maturity, and uniformity of spores. Additionally, quantitative methods, such as spore counting using a hemocytometer, were employed to ensure a statistically significant number of spores per unit area, with consideration given to a range of 10^5 to 10^6 spores per ml. These cultures were carefully stored at 4°C, primed for in-depth morphological, genetic, and pathogenicity studies.

2.2 Morphological assessment of the fungal isolate

2.2.1 Sample preparation and transfer

Under strict aseptic conditions, the fungal sample was transferred to a fresh Potato Dextrose Agar (PDA) plate using sterilized instruments. This process guarantees a contamination-free setting, preserving the natural morphological characteristics of the fungi.

2.2.2 Standardized incubation conditions

The newly inoculated PDA plates were carefully positioned inside a Biochemical Oxygen Demand (B.O.D) incubator, a standard for promoting fungal proliferation. The incubation continued for 7 days at a consistent temperature of 30°C. The timeframe was determined on the basis of empirical evidence, ensuring it's the best duration for the study of this fungal strain's morphological features.

2.2.3 Detailed colony morphology examination

Upon completion of the incubation, a thorough assessment of the fungal colony ensued. Observations were categorized based on expansion patterns, hue variations, margin intricacies, and notable surface topographies, which could be critical for taxonomic identification.

2.2.4 Microscopic morphological scrutiny

Using a Dewinter compound light microscope, we examined the shape and morphology of the conidial structures. This study focused on attributes like septation, form, size variations, and the identification of any potential vegetative structures like germ tubes and hyphae.

2.2.5 Rigorous spore dimensioning

To ensure statistical relevance, a broad spectrum of approximately 100 spores was methodically selected and assessed. By calculating the dimensions across this array, a reliable average spore size was ascertained, offering a robust metric for morphological comparison.

2.2.6 High-definition photographic cataloging

To retain a permanent record and for future references, high-fidelity microscopic images of the fungi were procured. Scale bars were represented for the microscopic images taken.

2.2.7 Data synthesis and documentation

Carefully collected observations and recorded images were methodically stored. The purpose is to establish a basis for comparing fungi and understanding the isolate's potential phylogenetic placement.

2.3 Pathogenicity test

In our pathogenicity testing aimed at assessing the potential harm to *R. ferrugineum*, a well-structured protocol was deployed. Pristine leaves of *R. ferrugineum* were chosen and placed onto sterilized Petri dishes under an aseptic environment to ensure the purity of the test. Gentle extraction of conidia from the mature fungal colony was done and suspended in autoclaved distilled water until a concentration of precisely 4.6×10^6 spores/ml was achieved. For inoculation, each *R. ferrugineum* leaf was treated with an exact volume of 100 µL of this conidial suspension, ensuring uniform exposure. One leaf that was intentionally left uninoculated served as the negative control, providing a basis for comparison. Following inoculation, the treated leaves were incubated under specific humidity-controlled conditions to create an optimal environment for any potential fungal proliferation. Post the incubation phase, these leaves underwent an intricate microscopic evaluation, employing the high-resolution Dewinter Excel Compound Light Microscope. This was essential to discern and capture the pathogenic interactions at the cellular scale. In accordance with scientific standards, we then re-isolate the fungus from prominent infection spots on the inoculated leaves. This was done in order to fulfill and validate Koch's postulates, an essential criterion in confirming the pathogenic nature of the organism. Concluding this procedure, the newly isolated fungal strain was juxtaposed with the initial field-obtained specimen. This exhaustive comparison gave insightful details about the plant-pathogen interaction and the threat they might pose.

2.4 Detailed genetic profiling through 18S-rRNA and its sequencing protocols

To comprehensively delineate the genetic lineage of the fungal isolate, we executed a meticulous genetic profiling strategy, employing well-established 18S-rRNA and ITS sequencing protocols. Commencing with the isolation of genomic DNA from the cultured fungal specimen, we subjected it to electrophoresis on a 1.0% agarose gel matrix to confirm its purity and integrity.

The visualization of a high-molecular-weight DNA band post-electrophoresis attested to the intact genomic DNA without degradation, reflecting the precision of our methodological approach. With confirmed DNA integrity, we progressed to the amplification stage, targeting the Internal Transcribed Spacer (ITS) region—an unequivocal genetic marker in fungal taxonomy. Successful amplification of this region was evident through a distinct amplicon band of approximately 600 base pairs (bp) on an agarose gel post-electrophoresis, employing a polymerase chain reaction (PCR) approach. To ensure the accuracy of our sequencing endeavors, purification of the PCR amplicon was paramount, eliminating residual primers, nucleotides, and potential contaminants. The pristine PCR product underwent bidirectional sequencing using ITS1 and ITS4 primers, executed with precision through the BDT v3.1 Cycle sequencing kit and ABI 3730xl Genetic Analyzer. Subsequent to sequencing, we navigated the realm of bioinformatics, aligning forward and reverse sequences into a refined consensus sequence through sophisticated aligner software. The consensus sequence underwent a BLAST search against the NCBI GenBank database, selecting the top 10 sequences with the highest similarity to our isolate's sequence.

To unravel the genetic relationship between our isolate and other fungal entities, we utilized ClustalW for multiple sequence alignment, creating a robust alignment of the selected sequences. This alignment served as the foundation for our subsequent phylogenetic analysis. Leveraging the capabilities of MEGA 10 software, we meticulously constructed a distance matrix and a phylogenetic tree, providing profound insights into the evolutionary lineage of our fungal isolate, as depicted in Figure 3 (Wang et al., 2014; Cheng et al., 2016).

2.5 Comprehensive phylogenetic examination and bioinformatic interpretation

In our study, the foundational data comprised the ITS sequences of 11 distinguishable *Curvularia* sp., including the newly characterized sequence of *C. tuberculata* OR262505.1 (elaborated in Table 1). Additionally, for phylogenetic interpretation, we utilized 18S rRNA genes. The ClustalW tool, was employed for this intricate genetic juxtaposition (Kumar et al., 2018). This methodical alignment ensures that our subsequent analytical procedures are rooted in precision, facilitating valid interpretations. Post alignment, we subjected the genetic data to an exhaustive evolutionary scrutiny using MEGA11 (Kumar et al., 2018).

In the subsequent critical phase of our study, we prioritized the Bayesian inference (BI) methodology. Using the PhyML+SMS software suite, BI generated a phylogenetic tree that not only depicted evolutionary trajectories but also maintained a foundation in statistical confidence (Lefort et al., 2017). The application of bootstrapping techniques provided our results with robust statistical support, ensuring that observed phylogenetic relationships among sequences were not coincidental but backed by empirical data. To present our findings in a visually accessible format, we utilized iTOL, a web-based application proficient in crafting interactive phylogenetic depictions (Trifinopoulos et al., 2016). This tool converted our raw data into an easy-to-understand phylogenetic tree, formatted in the widely accepted Newick format. Through this visual representation, we could clearly illustrate the evolutionary distances and complex genetic relationships among the studied *Curvularia* sp. isolates, offering stakeholders a comprehensive and detailed perspective on their genetic heritage (Dhara et al., 2020).

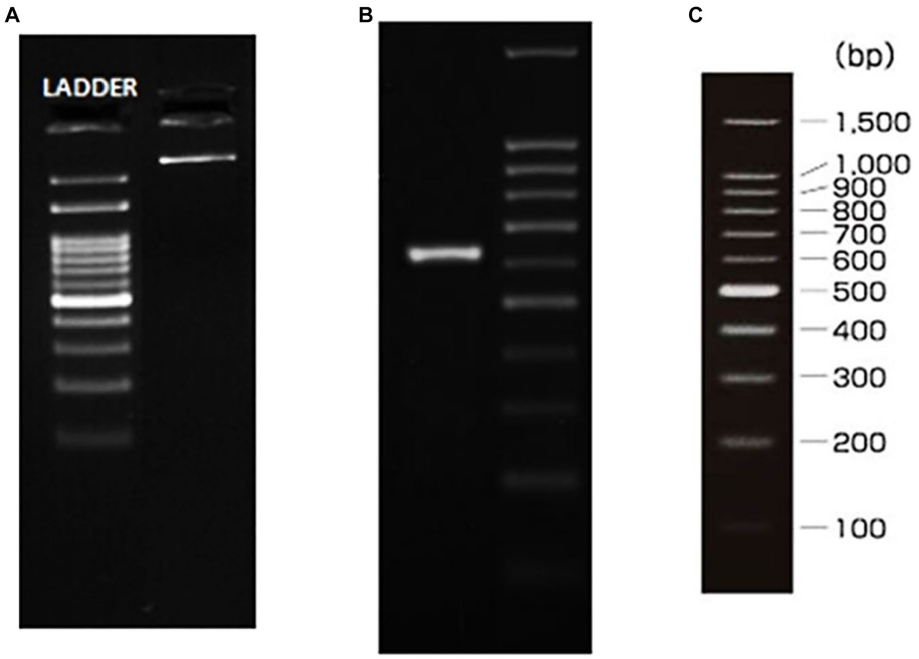

FIGURE 3
gDNA and ITS amplicon. **(A)** gDNA, **(B)** ITS PCR amplicon, **(C)** Ladder specification.

TABLE 1 Specifications of the isolates included in the phylogenetic study.

Species	GenBank accession	Isolate	Source	Geographical region
Curvularia tuberculata	LC494371.1	12,005	*Oryza sativa*	Taiwan: Tainan City, Liujia Dist.
Curvularia tuberculata	MF448225.1	FMB 0005	*Archontophoenix alexandrae*	Pakistan
Curvularia sp.	JN207337.1	P41E3	*Cyperus laevigatus*	Venezuela
Curvularia tuberculata	ON329689.1	e26	*Oryza sativa*	Mexico: Campeche
Curvularia tuberculata	HF934907.1	CBS 146.63	Collected genotyped isolates	Nebraska: Lincoln
Curvularia tuberculata	NR_138222.1	CBS 146.63	Collected genotyped isolates	Nebraska: Lincoln
Curvularia tuberculata	MN540245.1	L-3106/2012	Human corneal scraping	India
Curvularia tuberculata	MH665453.1	DWER3	*Datura wrightii*	India
Curvularia tuberculata	OR262505.1	RFP1	*Rhododendron ferrugineum*	India: Himachal Pradesh
Curvularia tuberculata	KJ767096.1	A1S2-D16	Beach soil	Malaysia
Curvularia sp.	JN207332.1	P36E3	*Eleocharis atropurpurea*	Venezuela

2.6 Cold stress-induced sporulation investigation

Initiated from a mature, 7-day old culture, the fungal specimen was meticulously translocated to dual PDA Petri plates, ensuring no external contamination. To simulate cold stress, these plates were methodically sequestered in a cold chamber, maintaining a consistent temperature of 4°C for a span of 8 days. Following this exposure period, a rigorous examination was undertaken. The fungal colonies were treated with Lactophenol cotton blue, a staining reagent renowned for its specificity to fungal structures. This staining regimen rendered the fungal morphology distinctly visible under the high-resolution optics of the Dewinter compound light microscope. To chronicle these observations and capture the nuanced details of sporulation, high-definition micrographs were obtained using a state-of-the-art Dewinter microscope digital camera, DIGI510, which boasts of a 5.1 MP 1/2.5´´CMOS sensor.

2.7 Culture media dependency on fungal growth dynamics

Recognizing the potential influence of culture substrates on fungal growth characteristics, an empirical study was designed to compare the performance of four distinct media formulations. The media chosen for this study encompassed: PDA (HIMEDIA M096, optimally suspended in distilled water at a concentration of 39 g/L), Malt Extract Agar (MEA) (HIMEDIA M1913, with a concentration of 61 g/L), Czapek Dox Agar (CZA) (HIMEDIA M075, meticulously formulated at 49.01 g/L), and Oat Meal Agar (OMA) (HIMEDIA M39, suspended at 72.5 g/L). To ensure uniformity in inoculation and negate potential variability, circular discs with a precise diameter of 6 mm were excised from a pristine, 7-day-old fungal culture utilizing 6 mm stainless steel sterilized cork borers. The discs for inoculation were placed at the center of sterile Petri plates containing the respective growth media. This setup was then incubated at a constant temperature of 30°C. Observations were recorded throughout the incubation period to monitor growth metrics and sporulation rate. Post incubation, for a detailed examination of sporulation patterns, samples were stained and observed under the Dewinter compound light microscope at a magnification of 400X.

3 Results & analysis

3.1 Comprehensive microscopic characterization of the isolated pathogen

Standard staining techniques and sophisticated, state-of-the-art microscopy tools were used to study the phenotypic and microscopic features of the fungal pathogen isolated from *R. ferrugineum* to determine their micro-morphological features. The deployment of Lactophenol cotton blue staining, renowned for its adeptness in accentuating the nuances of fungal configurations, in tandem with the high-definition capabilities of the Dewinter microscope digital camera (DIGI510, equipped with a superior 5.1 MP 1/2.5" CMOS sensor), yielded intricate insights into the pathogen's cellular architecture.

a) Conidial Structural Analysis: Microscopic observations revealed that the conidia exhibited a distinct distoseptate nature. Distinct septation patterns hint towards highly organized cellular mechanism/ s, which are an observable deviation from the conventional fungal septation patterns as shown in Figure 4.
b) Maturation and Septal Distinctions: During the maturation trajectory of the conidia, a notable morphological transformation was evident. Each septum bore darkened transverse bands, rendering a segmented appearance. The central cellular unit showcased heightened pigmentation compared to the terminal cells, potentially alluding to a concentration of vital organelles or compounds. Additionally, this central cell demonstrated a discernible dimensional advantage, being marginally voluminous.
c) Hyphal Dynamics: The fungal hyphae, essential components of the pathogenic structure, exhibited a subtle myelinization pattern. This myelinization, often linked to resilience and environmental adaptability, requires further biochemical exploration. An interesting observation was the distinct conidial attachment method, where a single conidium primarily adhered to the

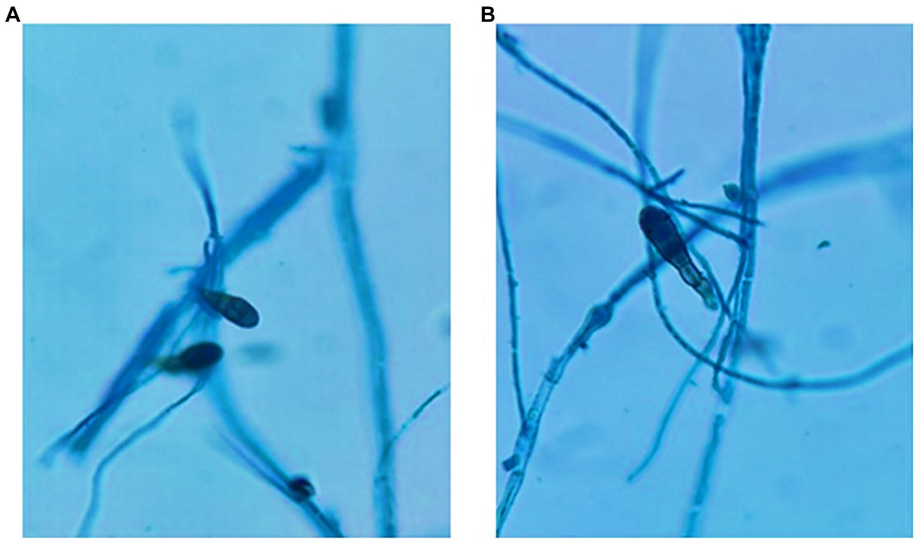

FIGURE 4
(A) and **(B)** Conidial structure of *C. tuberculata* after 7 days of growth in PDA, the fungal hyphae stained with Lactophenol cotton blue stain.

hyphae, indicating a strategic reproductive approach. Additionally, the stalk anchoring the conidium showed robustness, exceeding the thickness of the conidium's base. By examining these microscopic details, this analysis enhances our understanding of the pathogen's morphology and highlights the precision offered by contemporary microscopy and staining methodologies. Visual representations supporting these findings are available in Figure 4.

3.2 Multifaceted pathogenicity examination

The *in vitro* pathogenicity experiment was executed under rigorous conditions, revealing a panorama of intricate morphological changes on the leaves. Unraveling the landscape of the infection, the leaves exhibited patterns of lesions which ranged from irregular configurations to distinct ellipsoidal shapes. The lesions' borders emerged with a deep brown hue, encapsulating a gradient transition from buff to a grayish-brown on the lesion's surface. This particular manifestation potentially mirrors the systematic and aggressive colonization by the pathogen at various developmental stages. In stark contrast, control leaves, untouched by the fungal pathogen, retained their unblemished integrity even after incubation. The experiment involving the application of sterilized water served as a crucial control, confirming its innocuous nature by preserving the leaf's original morphology. Further microscopic scrutiny revealed more about the pathogen's nature. The ellipsoidal conidia, which is often an indicator of aggressive fungal strains, was accompanied by a subtle hilum, underscoring the unique morphological attributes of this pathogen.

3.3 In-depth insights from ITS sequencing

Embarking on the intricate journey into the pathogen's genetic blueprint, the ITS sequencing revealed a treasure trove of genomic revelations. Harnessing the power of the BLAST analysis, a striking genomic identity emerged. A near-perfect 99.82% similarity with numerous *C. tuberculata* strains and isolates underscores the taxonomic proximity and genetic fidelity of our isolated pathogen to known strains. Such a high degree of genomic alignment not only bolsters the identification but also accentuates the genetic consistency within this lineage.

3.4 Thorough phylogenetic dissection

Probing the evolutionary tapestry of the strains and isolates through an intensive phylogenetic analysis illuminated diverse genetic pathways and ancestral connections. Our analysis identified two sequences, OR262505.1 and RFP1, as the most closely related within the dataset. Although no bootstrap values were provided for this relationship, their proximity suggests a high degree of relatedness. A significant subtree within the phylogeny, rooted at MF448225.1 with a robust bootstrap value of 80, includes several Curvularia isolates. This subtree encompasses sequences JN207332.1 (bootstrap value: 48), which is closely related to JN207337.1 (bootstrap value: 58) and MH665453.1 (bootstrap value: 34), suggesting a moderately supported cluster. Another branch within this subtree contains ON329689.1 (bootstrap value: 31), which shares a close genetic relationship with LC494371.1 (bootstrap value: 42) and MN540245.1 (bootstrap value: 49), indicating a moderately supported group. NR_138222.1 and HF934907.1 are also closely related, displaying a bootstrap value of 76. KJ767096.1 is positioned as an outgroup, but no bootstrap support was provided, indicating that it is not closely related to the other sequences in the tree (Kimura, 1980). Figure 5 masterfully encapsulates these intricate genetic connections, weaving together a story of divergence, convergence, and co-evolution.

Collectively, this comprehensive exploration offers a holistic perspective on the pathogen's morphology, genetic identity, and

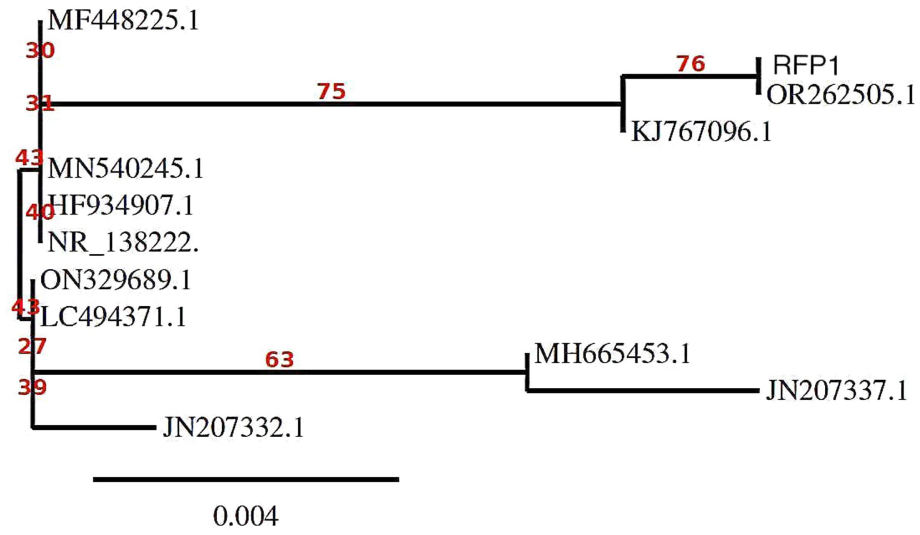

FIGURE 5
Molecular Phylogenetic analysis by Maximum Likelihood method using MEGA11 software.

evolutionary trajectory, presenting a detailed narrative that could be pivotal for future studies and interventions.

3.5 Cold-induced sporulation dynamics in fungi: an analytical exploration

To delineate the sporulation behavior of the fungal pathogen under a cold-stressed environment, a strategic experiment was instituted, ensuring a controlled yet replicable environment that mirrors potential natural climatic adversities (Wang et al., 2008).

a) Colony Dynamics under Cold Stress: Upon 6 days of methodical cold exposure, the ensuing colony dynamics exhibited a marked retardation in overall growth on the Potato Dextrose Agar (PDA) medium. This conspicuous deceleration likely signifies an orchestrated metabolic adjustment, a paradigm that several fungi adopt to economize energy expenditure and channel resources efficiently under non-ideal conditions.
b) Sporulation Proficiency Amidst Adversity: Despite the sub-optimal growth conditions, the fungal pathogen did not compromise its sporulation potential. The discernible presence of a substantive spore population underscores the pathogen's intrinsic reproductive resilience. The ability to prioritize sporulation even under adverse conditions is indicative of an evolutionary strategy to ensure species perpetuation.
c) Unperturbed Spore Morphology: In a significant observation, the spores, despite being subjected to cold stress, retained their morphological integrity. The cold environment, known to potentially disrupt cellular structures and fluidity in many organisms, appeared to have a minimal perturbative effect on these spores. Such robustness points towards a highly evolved cellular architecture, potentially fortified by protective bio-molecules or mechanisms that confer stability against environmental fluctuations. This morphological conservation is well-documented in Figure 6.
d) Interpretative Synthesis: The cold-induced sporulation dynamics suggests that while growth kinetics might be compromised, the fundamental reproductive modus operandi remains unscathed. The fungus appears to prioritize its long-term survival by investing in sporulation, even under stress. Such adaptive strategies are emblematic of organisms that have undergone evolutionary refinements over millennia, priming them for survival in diverse ecological niches.

The findings from this analysis illuminate a fascinating aspect of fungal adaptability and lay the groundwork for probing deeper into its cellular and molecular defense mechanisms. A more granulated study, possibly employing proteomic or transcriptomic analyses, could unravel the specific pathways and molecules at play during such cold stress responses. This, in turn, can have broad implications not just in ecology but also in applied sciences, especially in biotechnology and agriculture.

3.6 Impact of culture media on mycelial growth dynamics

The pivotal role of culture media in modulating fungal growth has always been well-acknowledged. Leveraging this understanding, a comprehensive exploration was undertaken to elucidate how varying culture environments might influence the growth and morphological dynamics of our test fungus.

a) Potato Dextrose Agar (PDA): This media emerged as the clear frontrunner, with the test fungi manifesting a prolific growth rate. With a mycelial expansion reaching an impressive 69 mm over a mere 6-day span, PDA's nutrient-rich composition seemingly offers an optimal environment, synergizing perfectly

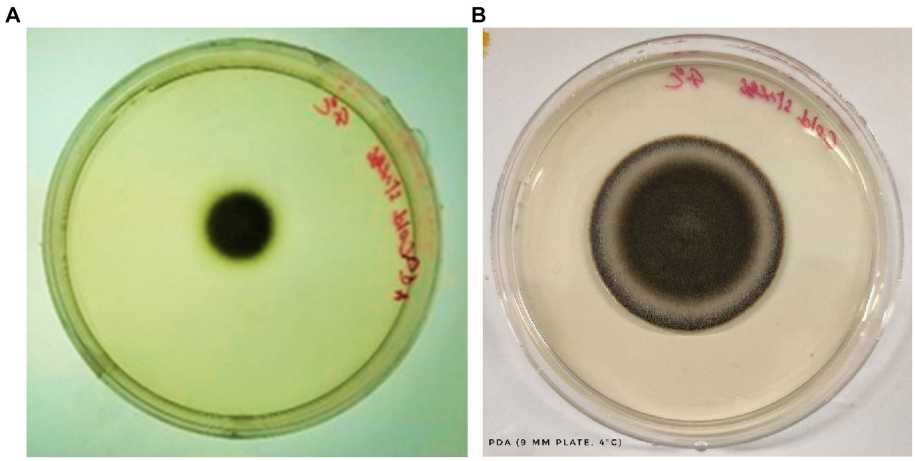

FIGURE 6
Growth of *C. tuberculata* under cold stress (4°C) after 6 days **(A)** and 8 days **(B)** of incubation, respectively.

with the fungi's metabolic requisites. This robust growth in PDA is suggestive of a conducive carbohydrate and mineral profile that aligns well with the fungi's physiological demands.

b) Oat Meal Agar (OMA) and Czapek Dox Agar (CZA): Both media exhibited commendable support for fungal proliferation. With growth metrics settling around 39 mm post a 6-day incubation window, it's evident that the nutrients available in OMA and CZA cater sufficiently to the fungi's growth algorithm, albeit not as effectively as PDA. While the exact constituents might differ, the growth suggests that the fundamental requirements of the fungi are being met in both media, leading to almost parallel growth statistics.

c) Malt Extract Agar (MEA): MEA, despite being a widely recognized medium for numerous fungi, displayed somewhat limited compatibility with our test fungus. Clocking in a growth of just 37 mm, this metric is a testament to the specific nutritional preferences or possible inhibitory components inherent in MEA for this particular fungus. However, a silver lining was the spontaneous and pronounced sporulation, hinting that MEA, despite not fostering aggressive vegetative growth, still offers conditions favorable for reproductive maturity.

d) Colorimetric Variations: The morphological canvas, especially the mycelial hue, presented significant diversities across the different media. Such variations could be attributed to differential nutrient assimilation or the influence of specific media constituents on fungal pigmentation pathways. These pigmentation differences not only serve as potential markers for media-specific growth conditions but might also provide insights into any associated physiological or metabolic shifts.

e) Sporulation Dynamics in MEA: Interestingly, while MEA trailed in promoting mycelial proliferation, it surged ahead in catalyzing sporulation. This intriguing dichotomy points towards a possible shift in the fungus's life strategy - prioritizing reproductive propagation over vegetative expansion when faced with certain nutritional constraints or stimuli.

The intricate interplay between culture media and fungal growth kinetics has been meticulously examined and vividly illustrated through the extensive analysis conducted in this study. Notably, the choice of culture medium exerts a profound influence on the behavior of the fungal pathogen under investigation, providing insights into its growth patterns and reproductive dynamics. One salient observation from our analysis is the prominence of Potato Dextrose Agar (PDA) as the optimal choice for stimulating sheer vegetative proliferation of the fungal pathogen. PDA, recognized for its nutrient-rich composition, provides an ideal environment for the pathogen's rapid vegetative growth. This medium's efficacy in promoting vegetative expansion highlights its utility when the primary research objective is to understand and quantify the pathogen's ability to proliferate and colonize host tissues. Conversely, our analysis also sheds light on the distinct propensity of Malt Extract Agar (MEA) to stimulate sporulation. MEA, with its unique nutrient composition and specific environmental conditions, serves as a catalyst for the fungal pathogen's reproductive processes. This medium facilitates the formation of spores, a critical aspect of the pathogen's life cycle. Researchers keen on exploring the reproductive dynamics, sporulation rates, or aspects related to the pathogen's life cycle would find MEA to be a valuable choice. In essence, this comprehensive analysis underscores the critical importance of deliberate media selection, contingent upon the specific research objective at hand. Whether the research focus centers on deciphering the nuances of vegetative growth, exploring the intricacies of reproductive dynamics, or encompassing both aspects, as outlined in Table 2 and visually represented in Figures 7, 8, the choice of culture medium becomes a pivotal determinant in shaping the trajectory and outcome of the study. It reinforces the notion that a well-informed decision regarding culture media is paramount in tailoring experiments to address specific research questions and objectives effectively, thus enhancing the depth and breadth of

scientific inquiry in the realm of microbial sciences (Table 2; Figures 7, 8).

4 Discussion

Microorganisms, those diminutive yet immensely influential life forms, occupy an irreplaceable niche within our ecosystems, weaving intricate connections between various facets of human existence and the broader environment. Recent findings, particularly in the domain of pathogenic fungi and their impact on *R. ferrugineum* plants, shed light on the significant implications of these microorganisms on our natural environment. In a thorough and exhaustive study, we have identified a notable issue drawing the attention of horticulturists, botanists, and plant pathologists alike — the emergence of prominent brown blotches on the leaves of *R. ferrugineum* plants. This can be attributed to the presence of the pathogenic fungus *C. tuberculata*, with specific emphasis on the exotype *Curvularia* sp. P41E3. These unsightly blotches, while diminishing the visual appeal of these plants, extend their repercussions beyond aesthetics. They impede the healthy development of flowers and leaves, thereby jeopardizing the ecological and economic significance of *R. ferrugineum* species. This fungal menace, hitherto known to afflict crops like rice and ornamental plants such as the Alexandra palm, has now, for the first time, been documented on *R. ferrugineum* L. plants within the Indian context. Our comprehensive research encompasses a spectrum of methodologies, ranging from the sequencing of the fungal ITS region to cultivating the pathogen in various fungal-specific media. These

TABLE 2 Mycelial growth, colony characteristics and sporulation pattern of test fungi on four different culture media.

Media type	Colony diameter (mm)		Colony character		Zonation	Sporulation
	4 days incubation	6 days incubation	Texture	Surface color		
CZA	27.5	39	Furry	Furry black center, basil green colored margin	Concentric	Heavy, conidia germinated
OMA	28	39	Cottony, raised in center	Cottony black colony raised in center, grayish color margin	Concentric	Moderate
MEA	26	37	Cottony	Cottony black colony	Concentric	Heavy, conidia germinated
PDA	58	69	Cottony, raised in center	Cotton black raised in center, creamy white margin	Concentric	Heavy
PDA (Cold Stress)	14	22	Cottony, raised in center	Cotton black raised in center, creamy white margin	Concentric	Heavy

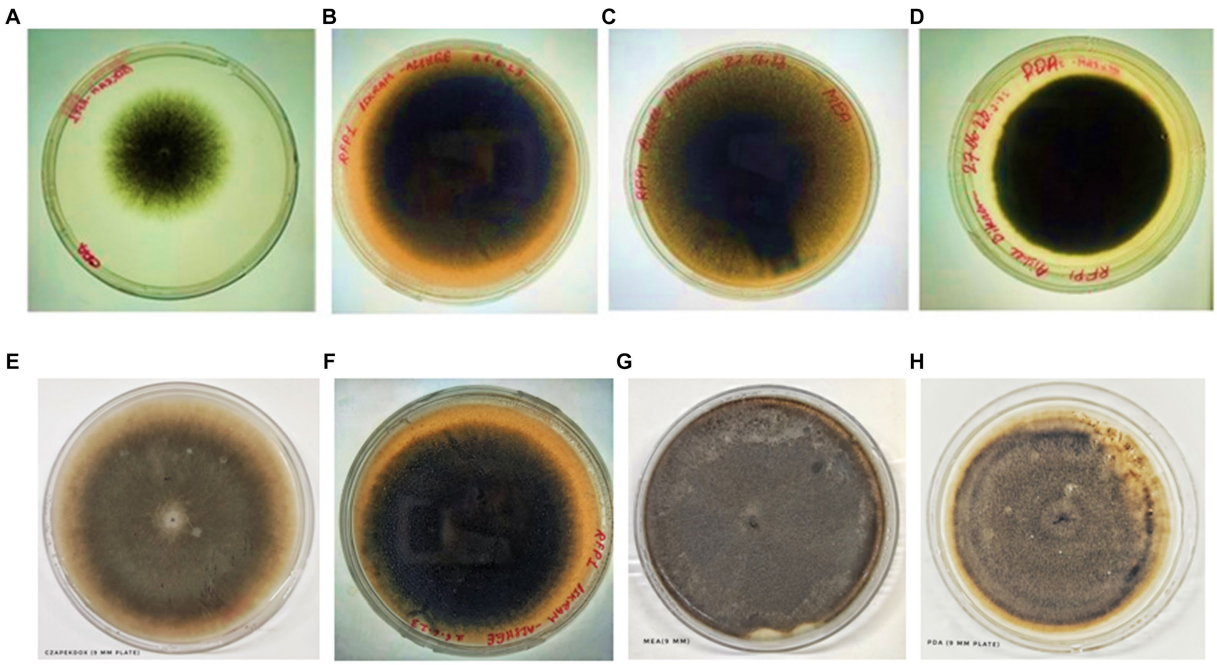

FIGURE 7
Colony of test fungi in CZA, OMA, MEA, and PDA, respectively, after 4 days of incubation **(A–D)** and 6 days of incubation **(E–H)**.

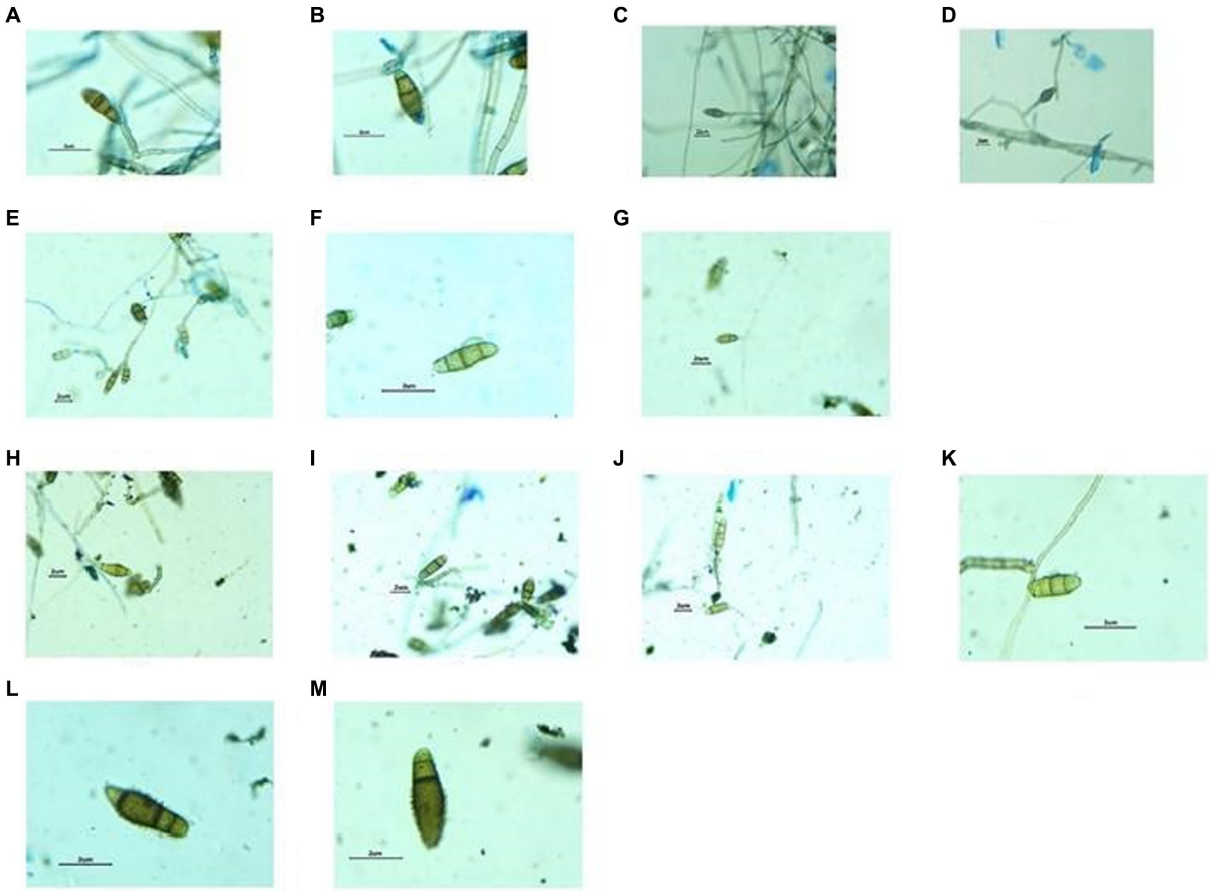

FIGURE 8
Structures of Fungal hyphae and conidia in different growth media under Dewinter compound light microscope and captured by DIGI510, 5.1 MP1/2.5 "CMOS sensor. Growth in CZA medium, Germinating conidia observed **(A–D)**, Growth in OMA medium, conidia slightly smaller in size **(E–G)** and Growth in MEA medium, Germinating conidia observed **(H–M)**.

efforts have yielded deeper insights into the characteristics of this fungus. After aligning the genetic data, we subjected it to thorough evolutionary analysis through MEGA11. This software, revered for its excellence in molecular evolutionary genetics analysis, provides researchers with the capability to explore genetic variances, trace lineages, and discern macroscopic evolutionary patterns inherent in the genetic makeup of the considered *Curvularia* sp. (Kimura, 1980).

In summary, our phylogenetic analysis of *Curvularia* sp. based on ITS sequences reveals distinct relationships among the isolates. OR262505.1 and RFP1 are closely related and likely form a distinct group within the phylogeny. The subtree rooted at MF448225.1 includes sequences with various degrees of relatedness, with moderate support for some relationships, such as JN207332.1, JN207337.1, and MH665453.1. The bootstrap values incorporated into the analysis provide a measure of confidence in the inferred relationships. Higher bootstrap values generally indicate stronger support for the depicted phylogenetic relationships. Therefore, our results suggest that MF448225.1, JN207332.1, JN207337.1, and MH665453.1 are closely related and share a common ancestor, while KJ767096.1 is more distantly related to the other sequences in the tree. These findings contribute to our understanding of the evolutionary history and relationships among *Curvularia* sp., which is valuable for their classification and ecological studies.

Particularly noteworthy is the robust growth exhibited by this pathogen, even in the face of cold temperatures, underscoring its adaptability and the formidable challenges associated with containing its proliferation. This discovery reverberates with the perspective championed at the United Nations General Assembly (UNGA) Science Summit — the imperative of comprehending microorganisms in their entirety. Whether the aim is to harness their beneficial potential or mitigate their adverse effects, the intricate world of microbes stands as a critical frontier of scientific inquiry. The United Nations Sustainable Development Goals (SDGs) resoundingly echo this sentiment, emphasizing the far-reaching influence of microbial sciences on domains as diverse as health, sanitation, agriculture, and more. Microbes, indeed, occupy a pivotal role in numerous sectors. In food production, they facilitate the generation of enzymes, pigments, and flavors, contributing substantially to culinary diversity (Raveendran et al., 2018). In agriculture, they bolster crop yields while concurrently reducing the detrimental impact of chemical inputs, exemplifying their ecological and economic significance. As the global community grapples with escalating health challenges, such as the emergence of new diseases and the paucity of effective drugs, microbes offer a beacon of hope. A multitude of current medications, including anticancer treatments and antibiotics, owe their existence to these microorganisms. The modern era has

witnessed their potential as biofactories for innovative drugs and proteins, owing to advancements in recombinant DNA technologies.

Moreover, microorganisms significantly contribute to industrial advancements by producing valuable substances and metabolites. Through omics-led microbial research, we can unlock their potential across diverse fields, from agriculture and health to environmental preservation. In essence, the world of microbes presents both immense opportunities and formidable challenges. While the pathogenic fungi affecting *R. ferrugineum* plants serve as a poignant reminder of the threats posed by microorganisms, the myriad of beneficial applications of these tiny powerhouses signifies their tremendous promise. By investing in a comprehensive understanding of microorganisms and harnessing their potential, we pave the way for a sustainable, prosperous, and harmonious future, where these minuscule entities become indispensable allies in our collective pursuit of global well-being and ecological preservation.

5 Conclusion & future perspective

The domain of microbial dynamics, owing to its foundational role within ecological networks, commands profound attention, particularly in light of imperatives surrounding sustainable development. This emphasis has been accentuated by recent deliberations at the United Nations General Assembly (UNGA) Science Summit. Recognizing the intricate interplay between microbial activity and global sustainability challenges, the UNGA Science Summit has ardently championed the intensive study and judicious harnessing of the microbial domain, aligning with the United Nations Sustainable Development Goals (SDGs). Our comprehensive investigation into the adaptive behaviors and growth characteristics of a specific fungal pathogen, within this expansive context, bestows a nuanced dimension upon our comprehension of the practical applications of microbial science. The discernible variations in growth patterns across a diverse array of culture mediums, coupled with the pathogen's remarkable resilience under stress conditions, serve as a microcosmic representation of microbial robustness and versatility. It is noteworthy that the escalating global emphasis on sustainable agricultural practices and the urgent need to reduce chemical inputs have propelled microbial solutions, such as biofertilizers, into the forefront of agricultural innovation. Our findings offer empirical insights that possess the potential to guide the development of such sustainable microbial interventions with precision and efficacy. Concurrently, within the realm of healthcare, where the world contends with an escalating trajectory of infectious diseases and a diminishing therapeutic arsenal, the phylogenetic nuances unearthed in our study portend promising avenues for targeted drug discovery and development. It bears emphasizing that the pharmaceutical repository is already enriched with microbial derivatives, further underscoring the sector's potential for groundbreaking innovation.

Beyond these specific domains, the broader implications of our study resonate with global challenges across a diverse spectrum. Whether pertaining to the assurance of water purity through microbial remediation or the harnessing of microbes for alternative energy solutions, our research's revelations regarding the fungal pathogen's adaptability symbolize the broader microbial capacity to respond to and potentially mitigate a wide array of ecological and societal challenges. In essence, our study, while delving into the intricate behaviors of a fungal pathogen, also serves as a compelling reminder of the untapped potential within the microbial domain. It provides empirical insights that not only enrich the ongoing scientific discourse but also chart pathways for future interdisciplinary explorations spanning ecology, agriculture, and healthcare. As we find ourselves at the confluence of numerous global challenges, the microbial world, with its vast potential, emerges not merely as an object of curiosity but as an indispensable ally in our unwavering quest for sustainable solutions.

The intricate relationship between microbial dynamics and ecosystem functionality, having only just begun to be unraveled, opens the door to expansive future exploration. The adaptive capacities exhibited by microbes, as exemplified in our study of a specific fungal pathogen, lay the groundwork for designing precise microbial interventions tailored to diverse environmental stressors. These interventions hold the potential to be pivotal in securing sustainable agricultural yields, especially in regions grappling with the rigors of extreme climatic conditions. Furthermore, the pharmaceutical arena holds substantial promise, with potential microbial pathways paving the way for innovative drug development and the formulation of strategies for disease mitigation (Jain et al., 2019). The challenges posed by antibiotic resistance and the increasing incidence of novel diseases underscore the urgency of these prospective endeavors.

Moreover, the burgeoning environmental challenges, spanning from wastewater management to plastic degradation, offer fertile ground for the development of microbe-assisted remediation techniques. The integration of advanced tools, including artificial intelligence and nanotechnology, stands poised to further propel microbial research, advancing us toward solutions that, at present, reside beyond our immediate purview. As we embark upon the new millennium, a resolute focus on microbial sciences promises not only to enrich our scientific repositories but also to equip humanity with innovative tools capable of effectively addressing the multifaceted global challenges that lie ahead. To fully realize this potential, continued dedication to fostering research in this field, coupled with interdisciplinary collaboration, is imperative, ensuring that the microbial world becomes an instrumental partner in our shared endeavor for a sustainable and harmonious future.

6 Limitations of the study

While our research has yielded valuable insights into the adaptive behaviors and growth characteristics of the specific fungal pathogen under investigation, it is important to acknowledge certain limitations inherent to our study. These limitations, rather than detracting from the significance of our findings, serve as points of departure for our future research endeavors.

6.1 Limited scope of culture conditions

Our study primarily explored the growth patterns of the fungal pathogen across a defined set of culture conditions. These conditions, although representative of key environmental stressors, may not encompass the full spectrum of conditions encountered in nature. Future research will aim to expand the range of culture conditions to provide a more comprehensive understanding of the pathogen's adaptability.

6.2 Limited application scenarios

While our study hints at potential applications in sustainable agriculture and pharmaceutical research, further empirical validation is needed to translate these findings into practical solutions. Future research will focus on rigorous field testing and clinical trials to ascertain the real-world applicability of our discoveries.

6.3 Temporal considerations

Our study represents a snapshot in time, and the adaptive behaviors of microorganisms can evolve over time. Conducting long-term studies to track changes in the pathogen's behavior and its implications on ecosystems is a valuable avenue for future research.

6.4 Single-pathogen focus

Our research centered on a specific fungal pathogen, offering valuable insights into its behavior. However, it is essential to recognize that ecological interactions often involve multiple species. Future investigations could delve into the dynamics of microbial communities and their responses to environmental stressors, providing a more holistic view of microbial ecosystems.

6.5 Interdisciplinary exploration

The multifaceted nature of microbial dynamics calls for interdisciplinary collaboration. Expanding our research to incorporate insights from diverse fields, such as genetics, ecology, and bioinformatics, will enrich our understanding of microbial behavior and its broader implications.

It is essential to view these limitations as opportunities for further investigation rather than as constraints. They pave the way for our next steps in research, guiding us towards a more comprehensive and nuanced understanding of microbial dynamics and their potential applications. Building upon the foundation laid by this study, our future research endeavors will address these limitations, ultimately contributing to a deeper and more impactful body of knowledge in the realm of microbial science.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GenBank, OR262502.1-OR262505.1.

Author contributions

JD: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. AH: Conceptualization, Data curation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. RP: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. VaK: -. VS: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft. ViK: Conceptualization, Data curation, Investigation, Writing – original draft. AM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – review & editing. AS: Conceptualization, Data curation, Investigation, Methodology, Resources, Writing – original draft. LA: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft. FE-D: Data curation, Investigation, Project administration, Software, Writing – original draft. MA: Investigation, Software, Validation, Writing – original draft. SA: Conceptualization, Investigation, Validation, Writing – original draft. MA-D: Investigation, Writing – review & editing. AK: Conceptualization, Methodology, Data curation, Software. BD: Conceptualization, Methodology, Software, Formal Analysis, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Function and therapeutic prospects of next-generation probiotic *Akkermansia muciniphila* in infectious diseases

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Akkermansia muciniphila is a gram-negative bacterium that colonizes the human gut, making up 3–5% of the human microbiome. *A. muciniphila* is a promising next-generation probiotic with clinical application prospects. Emerging studies have reported various beneficial effects of *A. muciniphila* including anti-cancer, delaying aging, reducing inflammation, improving immune function, regulating nervous system function, whereas knowledge on its roles and mechanism in infectious disease is currently unclear. In this review, we summarized the basic characteristics, genome and phenotype diversity, the influence of *A. muciniphila* and its derived components on infectious diseases, such as sepsis, virus infection, enteric infection, periodontitis and foodborne pathogen induced infections. We also provided updates on mechanisms how *A. muciniphila* protects intestinal barrier integrity and modulate host immune response. In summary, we believe that *A. muciniphila* is a promising therapeutic probiotic that may be applied for the treatment of a variety of infectious diseases.

KEYWORDS

next-generation probiotics, *Akkermansia muciniphila*, infectious diseases, intestinal flora, immune regulation

1 Introduction

The surface of human oral-gastrointestinal tract resides more than 100 trillion microorganisms including bacteria, fungi, parasites and viruses (Kamada et al., 2013). Researchers have found that gut microbiota has a complex and close relationship with human health and disease. Gut microbiota play critical roles in host immune regulation, inflammatory response, and energy metabolism. The disturbances or imbalances of gut microbiota are related to the development of a variety of diseases, such as inflammatory bowel disease (IBD),

metabolic syndrome, obesity, diabetes, and inflammation (Biedermann and Rogler, 2015). At present, there are more and more adverse reactions caused by the abuse of antibiotics, and the use of probiotics can reduce these adverse reactions, which brings new hope for human treatment and improvement of diseases. Probiotics currently used are several organisms conferring health benefit for the host.

In recent years, with the development of gut microbiome sequencing and strain isolation technology, new strains with potential health benefits were gradually found with no human applications, which are called the Next-generation probiotics (NGPs) (O'Toole et al., 2017). The development of NGPs is more likely aiming for pharmaceutical use than a food delivery route; hence, it can also be termed live biotherapeutic products (LBPs). Hence, there are some differences in the history and route to market between probiotics and NGPs.

Akkermansia muciniphila (*A. muciniphila*) is a next- generation probiotics with promising clinical application prospects, which is a resident of the human gut, making up 3–5% of the human microbiome (Derrien et al., 2004). It can grow in the intestinal mucus layer and feed on mucin secreted by the host, thereby colonizing the intestine through competitive rejection and protecting the intestine from pathogens. According to recent studies, *A. muciniphila* has shown beneficial effects on various fields including anti-cancer, delaying aging, reducing inflammation, improving immune function, regulating nervous system function, etc. Studies have also shown that oral doses of 1×10^{10} colony forming unit (CFU) in human volunteers are very safe, regardless of whether *A. muciniphila* bacteria are live or dead (Plovier et al., 2017). In 2021, the European Food Safety Authority confirmed the safety of pasteurized *A. muciniphila* and approved it for use as a novel food pursuant (EFSA et al., 2021). There is growing interest on the research of *A. muciniphila*, and many animal experiments confirm its positive roles in infectious diseases (Figure 1).

In this review, we provided a brief summary of the process on the basic characteristics and diversity of *A. muciniphila*, the impact and action mechanism of *A. muciniphila* and its derived components on infectious diseases, such as sepsis, virus infection, enteric infection and periodontitis. We also summarize the interaction mechanisms between *A. muciniphila* and host, focusing on the protection of intestinal barrier integrity and regulation of immune response and host metabolism.

2 Overview of *Akkermansia muciniphila*

Muriel Derrien et al. isolated a mucin-degrading bacterium from human feces and named it *Akkermansia muciniphila* in 2004, which is represented by the typical strain Muc^T (ATCC BAA-835) (Derrien et al., 2004). *A. muciniphila* is anaerobic and currently the only known member of the human intestinal *Vermicillae* residents. The cells of the Muc^T strain were oval-shaped, and the cell size varied with the culture medium. In the Muc^T medium, the diameter of strain Muc^T was 640 nm and the length was 690 nm. In BHI medium, the diameter of Muc^T strain was 830 nm and the length was 1 μm. Electron microscope shows filamentous structures in the surface of the bacteria growing in mucous media, which are capsular polymers used to connect cells and may contribute to bacterial adhesion and colonization in the gastrointestinal tract. The bacteria can grow at 20–40°C and pH 5.5–8.0, the optimum growth was at 37°C and pH 6.5 using mucin as sole carbon and nitrogen resource. Initial report indicated *A. muciniphila* is strictly anaerobic, whereas Ouwerkerk et al. reported that *A. muciniphila* is oxygen tolerant (Ouwerkerk et al., 2016). *A. muciniphila* can survive at nanomolar levels of oxygen, and the oxygen induced a complex transcriptional response of the bacteria by. *A. muciniphila* can survive for 48 h in ambient air, with a 25% survival rate after 24 h and with only 1% survival rate after 48 h.

A. muciniphila is present in distinct parts of the human mucosa and fecal samples based on 16S rRNA gene sequencing (Eckburg et al., 2005). It was also isolated from blood-culture sample of an 18-year-old woman (Dubourg et al., 2017), and the breast milk of healthy human (Hou et al., 2023). *A. muciniphila* presents at different stages of human life, whereas the levels varied between different ages and regions. *A. muciniphila* colonization starts in early life and increases to a level similar to adults (10^8 cells/g) within a year, whereas the abundance of the elderly decreased (Collado et al., 2007). Derrien et al. reported an

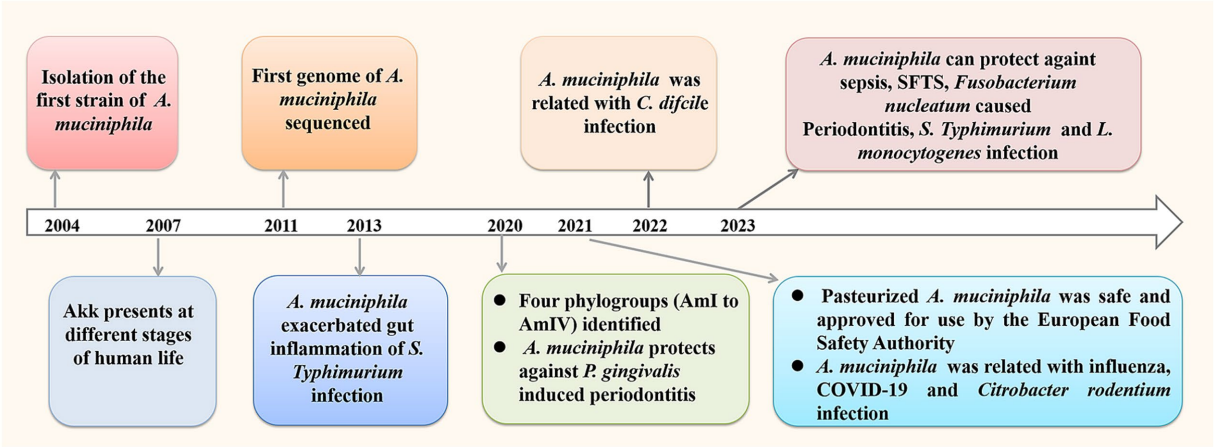

FIGURE 1
Research process of *A. muciniphila* with a focus on infectious diseases.

abundance of *A. muciniphila* over 1% (Derrien et al., 2008). Guo et al. reported that the colonization rate was 51.74% among the sample populationfrom southern China (Guo et al., 2016). *A. muciniphila* colonization rate in southern China was 51.71%, which was 74.70%in European populations. Considering influences of different regions, living environment and diet structure on microbial colonization, and further analysis of the correlation between age and *A. muciniphila* abundance is needed.

Mucin is the main component of human intestinal epithelium (Liévin-Le Moal and Servin, 2006). *A. muciniphila* can grow in the intestinal mucus layer and "feed" on mucin secreted by the host, and colonize in the intestinal tract through competitive rejection and protect the intestinal tract from pathogens. Although *A. muciniphila* uses mucin as an energy source, numerous observations have confirmed that *A. muciniphila* played positive regulatory function on the thickness and stability of intestinal mucus layer and the integrity of intestinal barrier.

3 Genome diversity and properties of *Akkermansia muciniphila*

A. muciniphila ATCC BAA-835 genome was revealed firstly in 2011 with 2, 176 protein-coding sequences (van Passel et al., 2011). Low identity (14.6–28.8%) between genomes of representatives of the *Verrucomicrobia* phylum was indicated. Through secretome analysis, 61 proteins are annotated as glycosyl hydrolases, proteases, sulfatases, and sialidases, which are candidates involved in the degradation of mucin. Using enterobacterial repetitive intergenic consensus (ERIC-PCR) DNA fingerprinting method, 12 distinct clusters were distinguished among 22 strains identified as *A. muciniphila* from southern China healthy human (Guo et al., 2016). *A. muciniphila* strains isolated from different people may belong to different subtypes and the strains with two different subtypes were isolated from the feces of a single subject. Further studies are needed to explain the relationship between subtypes of *A. muciniphila* and human health. Comparative genomic analysis also revealed that 23 *Akkermansia* strains could form four clades in phylogenetic trees (Xing et al., 2019).

In 2017, Guo et al. characterized the genomic architecture of *A. muciniphila* using whole-genome sequencing and the analysis of 39 human and mouse feces isolates and reconstructed 106 draft genomes from available metagenomic datasets, and they identified three phylogroups through phylogenetic analysis (Guo et al., 2017). Three species-level phylogroups (AmI, AmII, and AmIII) had distinct metabolic and functional features. AmI was the most frequent phylogroup, which were found in 93% of human samples, 91% of mice and 9% of pigs. AmII is also commonly found in the human gut, with a higher incidence in Europeans (44%) than Chinese (27%) and Americans (33%). In 2020, Kirmiz et al. reported four species-level phylogroups (AmI to AmIV) with distinct functions through comparative genomic analysis (Kirmiz et al., 2020). Genes for cobalamin (vitamin B12) biosynthesis were identified within the AmII and AmIII phylogroups, and vitamin B12 production by the AmII phylogroup were confirmed. Vitamin B_{12} is a crucial component in host–microbe interactions due to limited availability and its importance in the human gut (Degnan et al., 2014). Hence, the difference in vitamin B_{12} production for different phylogroup strains may indicate different interactions.

Population genomics analysis showed varied geographical and species distribution for different subspecies (Amuc1 to Amuc4) of *A. muciniphila*. A large-scale population genomics analysis was conducted for the *Akkermansia* genus including 188 sequenced genomes of the isolates and 2,226 genomes assembled from metagenomes of humans and other animals (Karcher et al., 2021). The results indicated that *A. muciniphila* showed whole-genome divergence and was stratified in four subspecies from Amuc1 to Amuc4, among which Amuc1 is most prevalent in humans (47%), followed by Amuc2 and Amuc3 (27 and 24%). Human specific Amuc2 and Amuc3 are not found in mice and non-human primates, whereas Amuc1 and Amuc4 are present in both humans and mice. The prevalence of Amuc4 was more commonly found in non-Westernized human populations compared to non-Amuc4 species. Analysis of metagenome-assembled genomes of *Akkermansia* revealed that Amuc III mainly distributed in the Chinese population and Amuc IV was more commonly present in Western populations, whereas Amuc I and II distributed extensively globally (Lv et al., 2022). The representative genomes of Amuc I, II, III, and IV showed diversified genomic characteristics involved in multiple metabolism and transport pathways, which suggests different evolution history and functional habits. Becken et al. proposed that AmI can be divided into two related subclades (Ia and Ib) (Becken et al., 2021). The doubling times of AmI strains was faster, while that of AmII and AmIV strains was slower. Strains also showed differences in their sensitivity to ambient oxygen, AmII was oxygen resistant and AmIV was very sensitive to oxygen. Different oxygen sensitivities were also observed in AmIa and AmIb groups. AmIb strains were highly sensitive to air exposure, whereas AmIa strains were moderate resistance to air exposure (Becken et al., 2021). The AmIV strain had high adhesion ability to epithelial cells and showed a greater tendency to aggregate when growing in mucin medium. Phylogroups AmIV and AmII outcompeted AmI strains in antibiotic-treated mice. Hence, the genetic and phenotypic diversity of *A. muciniphila* strains may be an important variable to consider when inferring the influences of this microbe on host health.

4 Antibiotic resistance characteristics of *Akkermansia muciniphila*

Antibiotic resistance of *A. muciniphila* is an important safety concern in its clinical application for disease treatments. Guo et al. compared the genomes of 40 *A. muciniphila* strains (39 newly isolates and ATCC BAA-835) with other genomes from the NCBI database, and reported the lateral gene transfer of eight genes between *A. muciniphila* GP36 and *Salmonella enterica* including three antibiotic resistance genes (Guo et al., 2017). These genes are *sul2* gene encoding sulfonamide-resistant dihydropteroate synthase, *aph(6)-Id* and *aph(3″)-Ib* gene encoding aminoglycoside phosphotransferase. Drug sensitive test was analyzed for *A. muciniphila* GP36 and ATCC BAA-835 including amikacin, sulfonamides, teicoplanin, polymyxin, cefoperazone-sulbactam, meropenem and minocycline. ATCC BAA-835 was resistant to teicoplanin and sensitive to other antibiotics, whereas *A. muciniphila* GP36 was resistant to amikacin, sulfonamides and teicoplanin. These results indicated the *A. muciniphila* might acquire antibiotic resistance via lateral gene transfer. Dubourg et al. reported that *A. muciniphila* Muc^T strain was susceptible to imipenem,

piperacillin/tazobactam and doxycycline, whereas was resistant to metronidazole (MIC >64 mg/L), vancomycin (MIC >64 mg/L) and penicillin G (MIC = 2.8 mg/L) (Dubourg et al., 2013). Antimicrobial susceptibility analysis indicated the resistance of *A. muciniphila* DSM 22959 (ATCC BAA-835) to chloramphenicol, clindamycin, streptomycin and erythromycin, whereas the strain was sensitive to ampicillin, tetracycline, gentamicin and kanamycin (Cozzolino et al., 2020). Machado et al. reported that *A. muciniphila* DSM 22959 strain was resistant to gentamicin, kanamycin, streptomycin (aminoglycosides) and ciprofloxacin (fluoroquinolones), whereas was susceptible to ampicillin, tetracycline, colistin, and fosfomycin (Machado et al., 2022). Opposite susceptibility results of gentamicin and kanamycin were reported for *A. muciniphila* DSM 22959 possibly due to different cut-off values and growth media used in the two studies.

Whether there is a risk of horizontal transfer of drug resistance genes is also an issue that needs to be considered in probiotic development. Machado et al. analyzed the genomes of 189 *A. muciniphila* strains and reported the existence of antibiotic resistance genes (ARGs) related with resistance to macrolides, fosfomycin, aminoglycosides, tetracyclines, and β-lactams (Machado et al., 2022). The ARGs of *A. muciniphila* DSM 22959 is consistent with the phenotypic feature for partial antibiotic resistance, while no resistant phenotypes were observed for the genes related with β-lactams, tetracyclines and fosfomycin resistance. Meanwhile, analysis of the genome sequences indicated that *A. muciniphila* DSM 22959 posed little risk of ARG horizontal transfer because there is no mobile genetic elements detected within its genome. Similar results were reported for the type-strain Muc^T and human isolates of *A. muciniphila*, intrinsic resistance genes observed seem to pose no risks by determining their antibiotic resistance phenotype, and there is no significant risk for the horizontal transfer of ARGs (Ouwerkerk et al., 2022). These results indicated the ARGs of *A. muciniphila* might pose a small risk of transmission.

Filardi et al. evaluated the antibiotic susceptibility of five human isolated *A. muciniphila* strains and found that one strain harboring *tetW* gene showing tetracycline resistance (Filardi et al., 2022). All five *A. muciniphila* strains had low sensitivity to ciprofloxacin and aminoglycosides including gentamicin, kanamycin and streptomycin, whereas no related antibiotic resistance genes were found in the genome. The gene *adeF* encoding one component of the resistance-nodulation-cell division efflux pump AdeFGH system was detected on the genomes of the isolates, whereas the treatment using efflux pump inhibitors did not alter the antibiotic susceptibility of the strains to ciprofloxacin. Hou et al. evaluated the safety of healthy human *A. muciniphila* isolates (AM01 to AM31) from feces and breast milk (AM06) as a probiotic (Hou et al., 2023). About 13 or 14 ARGs were predicted for AM01 to AM06 using Antibiotic Resistance Genes Database (ARDB), whereas Resistance Gene Identifier (RGI) analysis indicated only one antibiotic resistance gene in the genomes of AM01, AM04, AM05 and AM06, and two antibiotic resistance genes in the genomes of AM02 and AM03. The antibiotic susceptibility analysis indicated that all the strains were resistant to vancomycin, gentamicin, teicoplanin, ofloxacin, norfloxacin and bacitracin, whereas not all strains were resistant to cefoperazone, penicillin, and chloramphenicol, although they harbor related resistance genes. AM02 and AM03 showed resistance to lincosamide. In addition, AM01 and AM04 to AM06 showed resistance to kanamycin and ciprofloxacin, although no related antibiotic resistance genes were predicted. Hence, the presence of ARGs in the bacterial genome does not necessarily result in a resistant phenotype; more phenotype studies are required to confirm the antibiotic susceptibility of the strain especially for the new isolates.

In summary, current findings highlight the urgent need for standardized breakpoints and protocols to assess the antimicrobial sensitivity of *A. muciniphila* strains and to ensure comparability of results across different studies. Meanwhile, future studies should focus on clarifying the transferability risk of resistance genes. In addition, further studies on additional strains other than type strain of *A. muciniphila* are imperative to confirm the safety of this microbe in further application.

5 *Akkermansia muciniphila* and infection related diseases

5.1 *Akkermansia muciniphila* and sepsis

Sepsis is a life-threatening organ dysfunction caused by an unbalanced host response to infection and is a major medical burden worldwide (van der Poll et al., 2017). The pathogenesis of sepsis is closely related to intestinal flora, and clinical treatments for sepsis are still limited. Statistically, nearly 20% of deaths reported globally are due to sepsis (Rudd et al., 2020). *A. muciniphila* derived tripeptide RKH protects against lethal sepsis was reported recently and the mechanism was revealed (Xie et al., 2023) (Table 1; Figure 2). Compared with non-septic controls, gut *A. muciniphila* abundance in septic murine model significantly reduced. Interestingly, supplementation of both live *A. muciniphila* and its culture supernatant could significantly reduce the mortality of sepsis models. Live *A. muciniphila* and its supernatant could protect against sepsis associated organ damage and reduce pulmonary inflammation, whereas heat-killed *A. muciniphila* could not. Metabolomics analysis indicated elevated expression of a novel tripeptide Arg-Lys-His (RKH) in live *A. muciniphila* supernatant. Meanwhile, fecal RKH levels were significantly lower in patients with sepsis compared to healthy controls. RKH pretreatment could significantly extend the survival time of septic mice by alleviating acute tissue injuries and reducing inflammatory factor expression. RKH treatment suppressed the expression of proinflammatory cytokines in macrophages including bone marrow-derived macrophages (BMDMs) and human monocyte-derived macrophages (THP-1-dMs) after LPS stimulation. RKH inhibits systemic inflammation during sepsis through directly binding to the Toll-like receptor 4. Protection of RKH was also verified using a septic piglet model, and safety assessment indicated no obvious adverse effects *in vivo*. Hence, a novel tripeptide RKH produced by live *A. muciniphila*, may serve as a new promising treatment approach to combat lethal sepsis, which may need further evaluation before transformation into clinical practice.

5.2 *Akkermansia muciniphila* and virus infection

Influenza is a global infectious disease caused by a single stranded negative sense RNA virus named influenza virus, which is Liu et al.

TABLE 1 Reports on the function and action mechanisms of *A. muciniphila* on infectious diseases.

Diseases	Disease models	Forms of *A. muciniphila*	Effects	Action mechanism	References
Sepsis	Septic murine model; a septic piglet model	Live *A. muciniphila*, culture supernatant; tripeptide RKH	Alleviate acute tissue injuries and reduce sepsis-induced mortality	• Reducing inflammatory factor expression; • RKH inhibits systemic inflammation through directly binding to the Toll-like receptor 4 receptor.	Xie et al. (2023)
Influenza	H7N9 infection mouse model	Cultures and pasteurized *A. muciniphila*	Reduced weight loss and mortality	• Reducing IL-1β and IL-6 levels, enhancing IFN-β, IFN-γ, and IL-10 expression in H7N9-infected mice.	Hu et al. (2021)
COVID-19	COVID-19 patients	Live *A. muciniphila*	*A. muciniphila* abundance elevated in the COVID-19 patients	• *A. muciniphila* abundance positively correlated with inflammatory cytokines IL-1β and IL-6 and CXCL8.	Yeoh et al. (2021)
Severe fever with thrombocytopenia syndrome	SFTSV infection patients; Abx mice infection model	Live and pasteurized *A. muciniphila*	Akk abundance reduced in samples from deceased SFTS patients	• Reduced serum expression of IL-1β, IL-6 and TNF-α; • HAL regulates primary BA conjugation and protects host against SFTSV infection by suppressing NF-κB-mediated systemic inflammation.	Xie et al. (2023)
C. difcile infection	Mice infection model	Live *A. muciniphila*	Improve clinical outcomes of CDI mice	• Increased intestinal barrier by increasing the expression of tight junction proteins; • Reduced local and systemic immune response (reduced expression of IL-6, TNF-α, IL-1β, CCL1, CCL2, CCL3, CCL4, CCL5, CCL17, CCL22, CXCL10, and CXCL13); • Altered autophagy and innate immunity in the colon, alleviated microbiome dysbiosis, and improved bile acid and SCFA metabolism.	Wu et al. (2022)
C. difcile infection	Caco-2 cells	Live, UV-killed, cell-free supernatant and extracellular vesicles	Inhibition of cytotoxicity and inflammatory response	• Reduced the expression of IL-1β, TNF-α, and IL-10 in Caco-2 cell model; • Changed the expression of gut barrier–related genes and inflammatory response.	Nasiri et al. (2023)
Citrobacter rodentium induced colitis	Mice infection model	Live *A. muciniphila*	Reduce the symptoms and pathological changes	• Enhanced mucus barrier (upregulated expressions of gene encoding mucin, including muc1, muc5, and muc13) and anti-microbial responses (upregulation of Reg3γ, CRAMP and IL-22).	Mao et al. (2021)
Periodontitis (*Porphyromonas gingivalis*)	Mice infection model	Live, pasteurized *A. muciniphila;* Amuc_1100	Decreased periodontal destruction and systemic inflammation	• Increased anti-inflammatory effects (increased IL-10 and decreased IL-12); • Improved expression of tight junction molecules (ZO-1) and cell–cell adhesion markers; • Increased anti-infective response by upregulating IL-8 expression.	Mulhall et al. (2022) and Huck et al. (2020)
Periodontitis (*Fusobacterium nucleatum*)	Mice infection model	Live *A. muciniphila*	Inhibition of the periodontitis	• Inhibit TLR/MyD88/NF-κB pathway and secretion of inflammatory factors.	Song et al. (2023)

(Continued)

TABLE 1 (Continued)

Diseases	Disease models	Forms of *A. muciniphila*	Effects	Action mechanism	References
S. typhimurium infection	Gnotobiotic C3H mouse model	Live *A. muciniphila*	Exacerbating infectious and inflammatory symptoms	• Increased expression levels of IFN-γ, IP-10, TNF-α, IL-12, IL-17 and IL-6 in the cecal and colonic tissue of the mice.	Ganesh et al. (2013)
S. typhimurium infection	Streptomycin-treated C57B6J mouse infection model	Live and pasteurized *A. muciniphila*	Reduced fecal and systemic pathogen burdens and decreased inflammation responses	• AKK promotes the expression of intestinal barrier genes and the secretion of antimicrobial peptides. • pAkk promotes NLRP3 expression, and enhances the antimicrobial activity of macrophage through increased production of NO, ROS, and inflammatory cytokines.	Liu et al. (2023)
Listeria monocytogenes infection	High-fat/westernized diet mouse infection model	Live *A. muciniphila*	Reduce systemic infection	• *A. muciniphila* ameliorated inflammatory gene expression (decreased expression of TNFα and Foxp3, and elevated expression of Ccl2) in the distal ileum.	Keane et al. (2023)

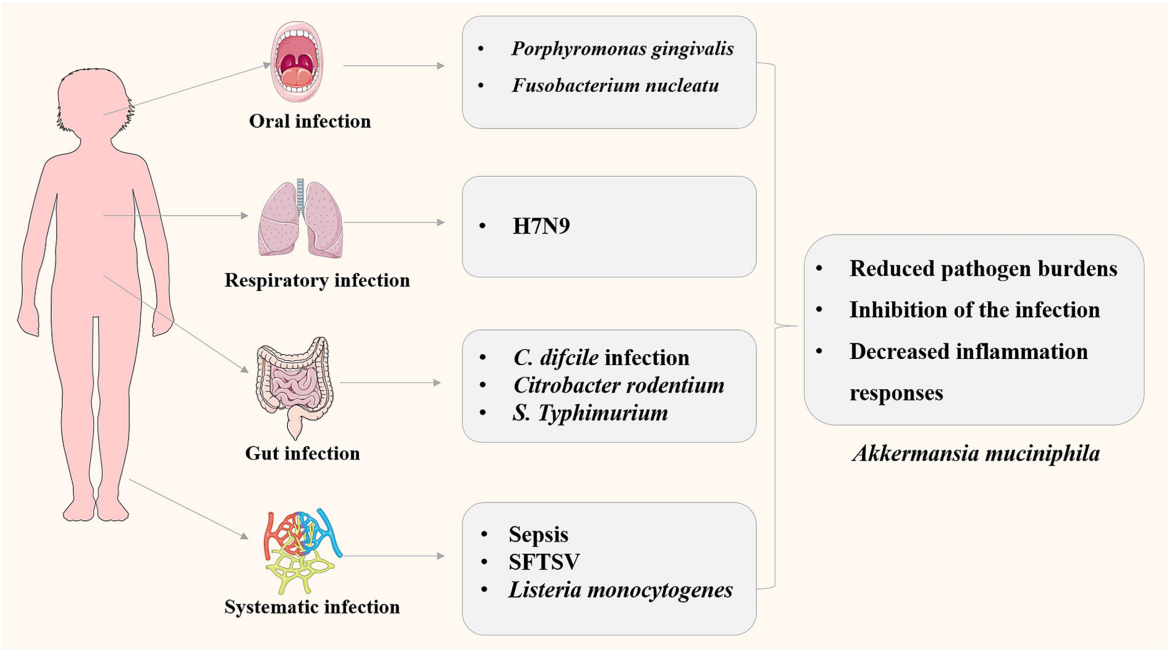

FIGURE 2
Roles of *A. muciniphila* in infectious diseases involving different pathogens and different systems.

(2023). According to the statistical data of World Health Organization, seasonal influenza epidemics cause an estimated 3 to 5 million cases of severe cases worldwide, resulting in approximately 500,000 deaths, and causing significant economic losses and social burdens (Eichberg et al., 2022). H7N9 influenza virus, an emerging zoonotic pathogen, has led to 1, 564 laboratory-confirmed cases of human infection from its initial outbreak until October 2017, and there are still sporadic infections now (Liu et al., 2023). Hu et al. reported that *A. muciniphila* improved host defense against influenza virus H7N9 infection (Hu et al., 2021). H7N9 infection could affect mouse gut microbiota including the increase of *A. muciniphila* abundance. Oral administration of pasteurized *A. muciniphila* (1×10^8 CFU) and its cultures significantly reduced weight loss and mortality of H7N9 infected mice, by reducing pulmonary viral titers, decreasing IL-1β and IL-6 levels, enhancing IFN-β, IFN-γ, and IL-10 expression. Hence, *A. muciniphila* could enhance host anti-influenza role through improving the innate immune response to H7N9 infection by regulating anti-inflammatory and immunoregulatory properties (Table 1).

COVID-19, caused by severe acute respiratory syndrome-Coronavirus 2 (SARS-CoV-2), is a pandemic that has affected the globe, leading to nationwide lockdowns. Research suggests that the gut microbiome may be a key factor in regulating host response and disease severity in COVID-19 patients (Aggarwal et al., 2022). Yeoh et al. analyzed the gut microbiota composition and host immune response markers in patients with COVID-19, and found that *A. muciniphila* abundance elevated in the COVID-19 patients (Yeoh et al., 2021). Moreover, the abundance of *A. muciniphila* positively correlated with inflammatory cytokines IL-1β and IL-6 and proinflammatory cytokine C-X-C motif ligand 8 (CXCL8). The cytokine storm was reported to be related with COVID-19 severity (Han et al., 2020). Hence, *A. muciniphila* may participate in the pathogenesis of COVID-19 and more researches are required.

Severe fever with thrombocytopenia syndrome (SFTS) is an tick-born infectious disease caused by a negative-strand RNA virus belonging to *phlebovirus*, which is originally reported in mainland China in 2009 (Yu et al., 2011). About 13, 305 patients have been diagnosed with SFTS in China until December 2020 (Che et al., 2022). Symptoms of SFTS include fever, thrombocytopenia, and leukopenia, with a fatality rate ranged from10 to 30% (Zhuang et al., 2018). Xie et al. reported an *A. muciniphila*–BA–TGR5 axis that regulates host NF-κB-mediated immunopathogenic responses to SFTSV infections (Xie et al., 2023). Relative abundance of *A. muciniphila* increased during the course of SFTSV infection in the surviving patients compared with heathy controls, whereas *A. muciniphila* abundance reduced in samples from deceased SFTS patients (SF-D group) compared with surviving SFTS patients (SF-S group). SF-S patients had reduced serum levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α compared with SF-D patients. The proinflammatory cytokine levels was inversely related with the relative *A. muciniphila* abundance. Intragastric administration of live and pasteurized *A. muciniphila* showed significant protection for Abx mice (microbiota-depleted mice) against fatal SFTSV infection compared with controls of unrelated commensal bacteria. The β-carboline alkaloid harmaline (HAL) generated by *A. muciniphila* can regulate primary BA conjugation and protect host against SFTSV infection through the inhibition of NF-κB-mediated systemic inflammation.

5.3 *Akkermansia muciniphila* and enteric infectious diseases

Clostridioides difcile (*C. difcile*) is a Gram-positive anaerobic bacterium, which can cause *C. difcile* infection (CDI) in healthcare facilities, with a high CDI recurrence rate of 15–35% (Finn et al., 2021). Gut microbe diversity is associated with the recurrence and severity of CDI (Maziade et al., 2015). Probiotics are widely recommended for the prevention of CDI and its recurrence, and the protective roles and underlying mechanisms of *A. muciniphila* on CDI were reported (Wu et al., 2022; Nasiri et al., 2023). Wu et al. reported that oral supplementation of *A. muciniphila* could reduce *C. difficile* burden and its toxins, and improve clinical outcomes of CDI mice including reduced body weight loss, alleviated diarrhea, relieved colon shortening (Wu et al., 2022). The protection mechanisms of *A. muciniphila* on CDI include increasing intestinal barrier by increased tight junction protein expression, reduced local and systemic immune response (reduced expression of IL-6, IL-1β, TNF-α, CCL1, CCL2, CCL4, CCL5, CCL3, CXCL10, CCL17, CCL22, and CXCL13), changing autophagy and innate immunity, alleviating microbiome dysbiosis, and improving bile acid and short-chain fatty acids (SCFAs) metabolism. The expression of autophagy related proteins (light chain 3 (LC3)-II, beclin1, autophagy-related gene 5 (Atg5), Atg9a, Atg7, and Atg12) and immune markers (, cluster of differentiation 14 (CD14), TLR4 and myeloid differentiation 88, MyD88) downregulated in the *A. muciniphila* group. Nasiri et al. reported that *A. muciniphila* and its derivatives could suppress cytotoxicity and inflammatory response induced by *C. difcile* RT001 *in vitro* using Caco-2 cells (Nasiri et al., 2023). Compared with untreated controls, the survival rate of Caco-2 cells treated with live, UV-killed, cell-free supernatant (CFS, 10^6 cfu/mL), and extracellular vesicles (EVs, 20 μg/mL) of *A. muciniphila* exceeded 90%. The treatment exerted function by reducing the expression of IL-1β, TNF-α, and IL-10 in Caco-2 cell model, and changing the expression of gut barrier–related genes and inflammatory response. Therefore, EVs and CFS of *A. muciniphila* may be a safe substitution to live bacteria that can prevent harmful effects of *C. difficile* toxins, which merits further *in vivo* verifications.

Protective roles of *A. muciniphila* were reported for another intestinal pathogen *Citrobacter rodentium*, which can induce bacterial colitis (Mao et al., 2021). Increased *A. muciniphila* abundance was correlated with the alleviation of *C. rodentium* infection and intestinal inflammation in the hyaluronan treated mice. *A. muciniphila* is the key species responding to hyaluronan treatment, and fecal transplantation experiments demonstrated the transferable of hyaluronan induced microbiome. *A. muciniphila* colonization in mice can significantly reduce the symptoms and pathological changes of *C. rodentium* infection, with less body weight loss, pathogen tissue loads and reduced proinflammatory cytokine (IL-1β) expression. The protective function of *A. muciniphila* on *C. rodentium* induced-colitis is implemented throughenhanced mucus barrier (upregulated mucin gene expression including *muc1*, *muc5*, and *muc13*) and anti-microbial responses (upregulation of Reg3γ, CRAMP, and IL-22). Together, these results indicate that *A. muciniphila* acts as regulator of gut barrier and immune responses to enteric infectious diseases.

5.4 *Akkermansia muciniphila* and periodontitis

Periodontitis, which occurs in the periodontal support tissue, is a chronic inflammatory disease that could lead to tooth loss in adults, and severe periodontitis has become the sixth most prevalent disease worldwide (Slots, 2017; Tonetti et al., 2017). *Porphyromonas gingivalis* is a gram-negative anaerobe and a main pathogen of periodontitis (Shalihin et al., 2023). It was demonstrated that oral gavage with pasteurized *A. muciniphila* decreased *P. gingivalis*-associated periodontal destruction and ameliorated systemic inflammation in lean and obese mice (Huck et al., 2020; Mulhall et al., 2022). Oral administration of *A. muciniphila* and its pili-like protein Amuc_1100 could reduce alveolar bone loss and inflammatory destruction in murine periodontitis models (Huck et al., 2020). *A. muciniphila* and *P. gingivalis* co-culture resulted in decreased expression levels of *P. gingivalis* virulence factor gingipains and increased expression of *A. muciniphila* Amuc_1100. The protection of *A. muciniphila* may act through increasing anti-inflammatory effects (increased IL-10 and

decreased IL-12) in Mouse bone marrow macrophages (BMMϕ), improving the expression level of tight junction molecules (ZO-1) and adhesion markers, and increasing anti-infective response by upregulating IL-8 expression in gingival epithelial cells. Interestingly, a similar protective effect was found for oral administration of pasteurized *A. muciniphila*, viable *A. muciniphila* and Amuc_1100 (Mulhall et al., 2022). The route of administration is key to preventing tissue destruction, as gavage does not significantly reduce periodontal destruction. The use of pasteurization *A. muciniphila* has more potential in an industrial point of view because it has minimal safety concerns and the same beneficial effects as live *A. muciniphila*.

Later, Song et al. reported an inhibition of *A. muciniphila* on the periodontitis caused by *Fusobacterium nucleatum* (Song et al., 2023). It has been verified that *F. nucleatum* can cause periodontitis and copolymerization with other periodontal pathogens, making it an important target for the prevention of periodontitis (Park et al., 2016). Bacterial co-culture experiments showed that *A. muciniphila* could restrain virulence gene expression of *F. nucleatum* by inhibiting TLR/MyD88/NF-κB pathway and inflammatory factor secretion. Finally, inhibition of *A. muciniphila* on the periodontitis caused by *F. nucleatum* was verified using BALB/c mice experiments. Therefore, *A. muciniphila* may act as a potential therapeutic strategy for periodontitis, which could inhibit the virulence factors of the pathogen causing periodontitis and reduce the immune response of the host.

5.5 *Akkermansia muciniphila* and foodborne infection

Nontyphoidal *Salmonella enterica subsp. enterica serovars* (*S. typhimurium*) is an intracellular bacterial pathogens causing hundreds of thousands of acute gastroenteritis cases each year (Vieira et al., 2022). There are two opposing reports about the effect of *A. muciniphila* on *S. typhimurium* infection in mouse model. Ganesh et al. reported that *A. muciniphila* exacerbated gut inflammation of *S. typhimurium* infection (Ganesh et al., 2013). A well-defined gnotobiotic C3H mouse model with a defined simplified human intestinal microbiota (SIHUMI) of eight bacterial species was used to analyze the influence of *A. muciniphila* on inflammatory and infectious symptoms caused by *S. typhimurium*. Additional *A. muciniphila* colonization in *S. typhimurium*-infected C3H mouse model significantly elevated histopathology scores and increased expression levels of IFN-γ, TNF-α, IL-12, IP-10, IL-17, and IL-6 in the cecal and colonic tissue of the mice, thereby exacerbating infectious and inflammatory symptoms. However, Liu et al. reported that *A. muciniphila* could decrease mice susceptibility to *S. typhimurium* infection (Liu et al., 2023). A streptomycin-treated C57B6J mouse infection model was constructed to evaluate the impact of live *A. muciniphila* (AKK) and pasteurized *A. muciniphila* (pAKK) on *S. typhimurium* infection. The results indicated that AKK and pAKK pretreatment significantly reduced pathogen burdens and decreased inflammation responses during *S. typhimurium* infection. There may have different protective mechanisms for AKK and pAKK treatments. Analysis of SCFAs levels indicated higher propionate levels in AKK-treated group compared to control or pAKK-treated mice. Live *A. muciniphila* promotes the expression of intestinal barrier genes and antimicrobial peptide secretion, and co-housing studies have shown that *A. muciniphila*-associated microbial communities play a role in alleviating infection symptom. The researchers verified that pAKK pretreatment could promote NLRP3 expression, and increase the antimicrobial activity of macrophage, possibly through increased production of nitric oxide (NO), reactive oxygen (ROS) and inflammatory cytokines. Differences between the composition of microbial community and infection conditions in mice could explain the opposite phenotypes observed between two studies, and further research is required to reveal the roles and mechanisms of *A. muciniphila* in *S. typhimurium* infection.

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes mild to severe gastroenteritis in healthy individuals, whereas can cause bacterial sepsis, bacterial meningitis in children, elderly individuals, and immunocompromised individuals (Radoshevich and Cossart, 2018). *L. monocytogenes* is rod-shaped facultative anaerobes, which can tolerate low temperatures and has high resistance to environmental stresses, making it a major concern for the food industry (Gandhi and Chikindas, 2007). The effects of *A. muciniphila* on *L. monocytogenes* infection in the high-fat/westernized diet mice were investigated (Keane et al., 2023). *A. muciniphila* treatment could reduce systemic *Listeria* infection induced by diet by reducing the bacterial loads in the liver, spleen and mesenteric lymph nodes, demonstrating that oral gavage with *A. muciniphila* enhances mice *L. monocytogenes* resistance. The molecular mechanism may be that *A. muciniphila* ameliorated inflammatory gene expression (decreased expression of Tnfα and Foxp3, and elevated expression of Ccl2) in the distal ileum thus leading to a reduction in inflammatory cell infiltration. The results indicated potentials for the use of microbial interventions in the prevention of foodborne infectious diseases. The roles of *A. muciniphila* in infection related diseases were summarized in Figure 2.

6 Interaction mechanism between *Akkermansia muciniphila* and host

6.1 Regulation of host immune response and inflammation

Intestinal immune barrier refers to innate and adaptive immune cells and gut-associated lymphoid tissue colonized on the intestinal lamina propria (Di Tommaso et al., 2021). Environmental factors, intestinal flora and their metabolites could be recognized by specific receptors (toll-like receptors, TLRs) on immune cells, leading to intestinal immune homeostasis or imbalance (Shi et al., 2023). Multiple investigations have shown that *A. muciniphila* plays an important role in regulating the immune response and host inflammation. The regulatory roles could complete by a variety of forms such as live or inactivated bacteria, culture supernatant or derived components of *A. muciniphila*. Germ-free mice were utilized to analyze the influence of *A. muciniphila* on the host response (Derrien et al., 2011). *A. muciniphila* colonized most in the cecum, which produce the most mucin. Global transcriptional analysis indicated that *A. muciniphila* changed mucosal gene expression profiles by increasing the expression of genes involved in immune responses and cell fate. Exposure to SCFAs generated by *A. muciniphila* could alter the gene transcriptional levels in mouse ileal organoid

model (Lukovac et al., 2014). The results indicated that *A. muciniphila* and its metabolites had an impact on the expression of transcription factors and genes involved in cellular lipid metabolism and growth. The β-carboline alkaloid harmaline (HAL) generated by *A. muciniphila* can regulate primary BA conjugation and suppressed NF-κB-mediated systemic inflammation (Xie et al., 2023). Oral administration of cultures and pasteurized *A. muciniphila* (1×10^8 CFU) significantly reduced IL-6 and IL-1β levels, enhanced IFN-γ, IFN-β, and IL-10 expression in H7N9-infected mice (Hu et al., 2021). The inhibitory effects of *A. muciniphila* and its derivatives on cytotoxicity and inflammatory response were also reported for *C. difcile* RT001 induced Caco-2 cells (Nasiri et al., 2023). In summary, protective effect *of A. muciniphila* and its derivatives is inversely associated with inflammatory status and aids the immune response by regulating anti-inflammatory pathway (Figure 3).

Adaptive immune cells play an important role in the protection of intestinal mucosal barrier and tissue homeostasis through immunoglobulin A (IgA) (Belkaid and Harrison, 2017). Mouse studies have shown that *A. muciniphila* can specifically induce immune responses to T cells during homeostasis (Ansaldo et al., 2019). *A. muciniphila* has been shown to induce IgG1 and T cell-related immune responses in mice, T-cell response induced by *A. muciniphila* could also occur independently through follicular T-cell. Amuc_1100 protein is a pili-like protein highly abundant in the outer membrane of *A. muciniphila* Muc^T, which could activate the NF-κB pathway through activation of receptors such as Toll-like receptor (TLR) 2 and TLR4 (Ottman et al., 2017). Recently, a phospholipidlipid was identified from the cell membrane *A. muciniphila*, with immunomodulatory activity in cell-based assays through toll-like receptor TLR2–TLR1 heterodimer (Bae et al., 2022). The phospholipidlipid could induce pro-inflammatory cytokines IL-6 and TNFα expression. Moreover, it can reset the activation threshold of dendritic cells and regulate the immune stimulation. Dendritic cells typically recognize and respond to bacterial metabolites through the pathogen-associated molecular pattern (PAMP) receptors such as TLR2 and TLR4 (Kang et al., 2009).

Besides T cells, *A. muciniphila* can also influence the intestinal immune through regulating the function of other immune cells (Figure 3). *A. muciniphila* derived tripeptide RKH could suppress the expression of proinflammatory cytokines in macrophages after LPS stimulation and directly bind to TLR4 and inhibit systemic inflammation of sepsis (Xie et al., 2023). Treatment of peripheral blood mononuclear cells (PBMCs) with *A. muciniphila* (live cells, heat-killed cells and supernatant) induce production of both anti-inflammatory and pro-inflammatory cytokines (IL-6, IL-1β, IL-8, IL-10, and TNF-α) (Ottman et al., 2017).

6.2 Enhancement of the intestinal barrier function

The intestinal barrier is a complex and well-organized physiological structure, which interacts with the external environment as a biochemical, physical, and immune barrier (Breugelmans et al., 2022). In healthy conditions, the intestinal barrier is semi-permeable, allowing the absorption of nutrients and water and protecting the internal environment from potential penetration by pathological molecules and microorganisms (Maynard et al., 2012). However, the damage of the intestinal barrier integrity results in multiple local and systemic diseases. Intestinal mucus consists of an inner layer without bacteria and a thicker outer layer with symbiotic bacteria (Hansson and Johansson, 2010). The integrity of the intestinal barrier requires normal epithelial boundary, maintenance of tight junctions, normal mucus secretion and a normal gut microbiome, as well as a finely regulated immune system (Shi et al., 2023).

Mucins, composed of amino acids and oligosaccharides, is a nutrient source for intestinal bacteria anda gatekeeper of the gastrointestinal mucosal barrier (Breugelmans et al., 2022). *A. muciniphila* bacteria can grow using mucins as sole carbon and nitrogen source in the intestinal mucus layer, and settle in the intestinal tract through competitive rejection and protect the intestinal tract from pathogens (Figure 3). The presence of mucin could increase the expression levels of mucin-degrading enzymes, whereas most genes involved in glycolysis and energy metabolic pathways upregulated under low mucin conditions (Shin et al., 2019). *A. muciniphila* abundance was positively related with mucin content in the cecum (van den Abbeele et al., 2011). Antibiotic treatment increased the mucus barrier by reducing the abundance of mucin-degrading *A. muciniphila* and decreasing Muc2 gene (encoding the major mucin of the colonic mucus in colonic tissues) expression (Ijssennagger et al., 2015). Mucin levels in the small intestine increased indicated by increased expression of MUC5 and MUC2 after metformin treatment in female mice, and thickened intestinal mucosa was confirmed by immunohistochemical assays and *A. muciniphila* abundance increased (Lee and Ko, 2014). *A. muciniphila* abundance was positively associated with the number of mucin-producing goblet cells in mice after metformin treatment (Shin et al., 2014). *A. muciniphila* also simulates mucin production in addition to its ability to degrade mucin. Therefore, although *A. muciniphila* make use of mucin as an energy source, it can positively regulate the thickness and stability of intestinal mucus layer and the intestinal barrier integrity.

In addition to affecting the mucin layer, other mechanisms may affect the integrity of the intestinal barrier (Figure 3). The interaction between *A. muciniphila* and the host might influence immune tolerance and homeostasis in the gut (Everard et al., 2013). Viable *A. muciniphila* administration elevated the levels of intestinal endocannabinoids and improved the metabolic profile and the mucus layer thickness (Everard et al., 2013). Endocannabinoids play roles in the gut inflammation, gut peptide secretion and the gut barrier. *A. muciniphila* and Amuc_1100 increased trans-epithelial resistance (TEER) in Caco2-cells, indicating their roles in strengthening the epithelial barrier function (Ottman et al., 2017).

Tight junctions are the connections between intestinal epithelial cells, which consist of zonula occluden (ZO), claudins (Cldns) and occludin (Ocln) proteins, are critical for normal function of epithelial cells and maintenance of intestinal barrier functions (Allam-Ndoul et al., 2020). *A muciniphila* could increase the expression of intestinal tight junction proteins including ZO-1 and occluding proteins, thereby decreasing Western diet-induced gut permeability and contributing to the gut barrier function (Li et al., 2016). Luo et al. reported that active or autoclaved *A. muciniphila* could induce the expression of tight junction proteins (ZO-1 and occluding proteins) in intestinal epithelial cells (Luo et al., 2021). The apoptosis rate can reflect the degree of cellular damage, and *A. muciniphila* treatment significantly suppressed the apoptotic rate of the inflammatory

IPEC-J2 cells, thus reducing the degree of cellular damage. Therefore, *A. muciniphila* may protect intestinal barrier integrity by regulation of epithelial cells, mucus secretion, tight junctions, normal gut microbiota and cellular damage level.

6.3 Metabolic regulation and nutrition of the intestinal wall

A. muciniphila genome encodes a large number of mucin-degrading enzymes, which can degrade mucin and generate short-chain fatty acids, such as butyrate, propionate, acetate, etc., playing important roles in the regulation of host metabolism and disease development (Cani and de Vos, 2017). Live, pasteurized bacterium and Amuc_1100 protein could improve metabolism in obese and diabetic mice (Plovier et al., 2017). Interestingly, pasteurized *A. muciniphila* showed stronger impacts on glucose intolerance, body weight and fat mass gain and in HFD-fed mice, which was associated with modulation of host urinary metabolomics and energy absorption of the intestinal tract. Everard et al. reported the roles of *A. muciniphila* in metabolic regulation including adipose tissue metabolism, fat storage and glucose metabolism in diet-induced obesity mouse model (Everard et al., 2013). *A. muciniphila* therapy completely reversed diet-induced fasting hyperglycemia via decreasing hepatic glucose-6-phosphatase expression, and affected adipose tissue metabolism by increasing the mRNA expression of adipocyte differentiation and lipid oxidation markers. Meanwhile, the colonization of *A. muciniphila* in IFNγKO and wild-type mice can improve host glucose metabolism was reported and verified in humans (Greer et al., 2016). IFNγ may control gut *A. muciniphila* levels by regulating Irgm1 gene expression in the mouse ileum. The relation between IFNγ, *A. muciniphila* and glucose tolerance exists in humans, suggesting a conserved mechanism in the regulation of metabolic health in mice and humans. Hence, *A. muciniphila* played roles in host metabolism regulation including glucose and lipid metabolism (Figure 3).

7 Issues to be considered in the application of *Akkermansia muciniphila*

The general characteristics of *A. muciniphila* strains from different origins, the efficacy of the strain on the diseases, the antibiotic resistance and the toxicity on the host should be evaluated completely before the application of *A. muciniphila*. Most reports on *A. muciniphila* treatment are conducted *in vitro* or using animal model, hence clinical trials must be conducted to confirm the safety and the efficacy of this promising NGP. Different studies may have reported opposite therapeutic effects, such as the effect of *A. muciniphila* on *S. typhimurium* infection (Ganesh et al., 2013; Liu et al., 2023), which is also worth further research to verify. The phenotype of antibiotic resistance to and the possible horizontal transfer of resistance genes also need more research. The resistance of different *A. muciniphila* strains to different antibiotics was different. Different susceptibility results of gentamicin and kanamycin were reported for *A. muciniphila* DSM 22959 due to the reference to

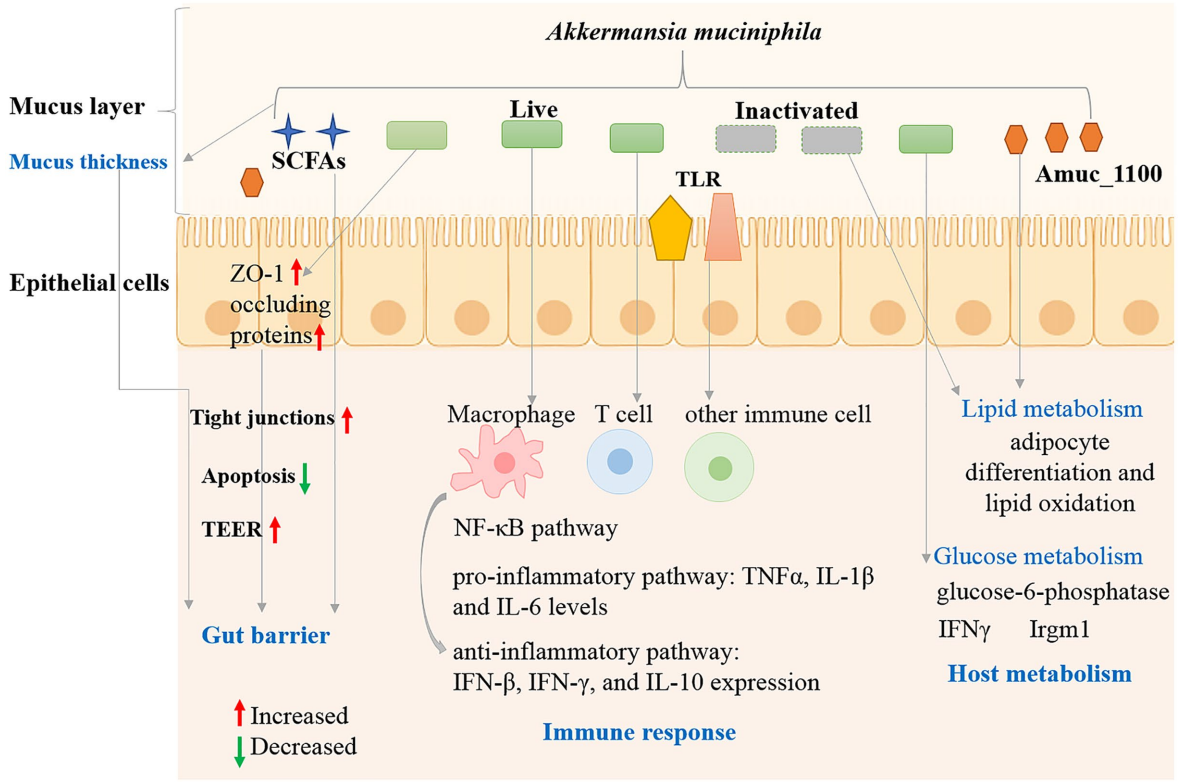

FIGURE 3
Interaction mechanisms between *A. muciniphila* and host.

different cut-off values and the methods used (Cozzolino et al., 2020; Machado et al., 2022). Hence, there is urgent need for standardized protocols and breakpoints to evaluate the antimicrobial sensitivity of *A. muciniphila* strains. More researches are required to access the antibiotic resistance of *A. muciniphila* strains isolated from different humans and different samples besides the type strain. One aspect is that as a gut microbe *A. muciniphila* starts to colonize in healthy subjects early in life and is also detected in breast milk and blood of human (Collado et al., 2007; Dubourg et al., 2017; Hou et al., 2023). Increased abundance of *A. muciniphila* may affect different systems in different individuals. The use of *A. muciniphila* derived bioactive molecule may be an alternative. In addition, the regulatory approval for NPG is also a concern in the drug development. In the US, the definition of live biotherapeutic products (LBP) presented by Food and Drug Administration (in 2012) overlaps NGPs (O'Toole et al., 2017). NGPs are not considered as microorganisms intended for human use so far. In Europe, the European Pharmacopoeia includes a class of products intended to prevent or treat diseases as medicinal products since 2019, which need to be registered under the rules for newly developed drugs (Sionek et al., 2023). This means that before application of NGPs, there should be a series of clinical trials (phase 1–3) to determine safety, dose range, side effects, and benefits. Hence, before *A. muciniphila* can be used in clinical treatment, there are still issues that require to be determined by further researches to ensure the safety and efficacy of treatment, and to obtain the approval for human-use.

8 Conclusion

A. muciniphila is a current star in the research field of next-generation probiotic because it colonizes in the mucus layer, a niche close to host cells, where it plays crucial role in gut homeostasis, exhibiting beneficial effects on several pathologies. *A. muciniphila* could produce small metabolites and mediators, affect microbial diversity and protect gut barrier integrity, thus exerting beneficial impacts on the gut and regulating a series of diseases including the metabolic, cardiovascular, infectious, and neurological diseases. The protective roles of *A. muciniphila* were reported for different infectious diseases including oral infection, respiratory infection, gut infection and systematic infection involving different pathogens such as bacteria and virus. The protection mainly act through reducing pathogen burdens, inhibition of the infection symptoms, and decreasing inflammation responses. Although preliminary data of the novel probiotic in the infectious diseases were inspiring. Further studies are required to reveal the exact roles of *A. muciniphila* in these areas and confirm the safety of *A. muciniphila* treatment, in particular to compare the effects of live, pasteurized and critical components of *A. muciniphila*, to test the efficacy of the protection roles in human beings.

Author contributions

LL: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. ML: Formal analysis, Writing – review & editing. YC: Supervision, Writing – review & editing. ZeY: Investigation, Writing – review & editing. PC: Data curation, Writing – review & editing. ZhY: Validation, Writing – review & editing. WC: Supervision, Writing – review & editing. WZ: Supervision, Writing – review & editing. ZW: Resources, Writing – review & editing. XG: Formal analysis, Investigation, Writing – review & editing. HS: Data curation, Supervision, Writing – review & editing. XW: Investigation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Whole genome sequencing and characterization of *Pantoea agglomerans* DBM 3797, endophyte, isolated from fresh hop (*Humulus lupulus* L.)

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Background: This paper brings new information about the genome and phenotypic characteristics of *Pantoea agglomerans* strain DBM 3797, isolated from fresh Czech hop (*Humulus lupulus*) in the Saaz hop-growing region. Although *P. agglomerans* strains are frequently isolated from different materials, there are not usually thoroughly characterized even if they have versatile metabolism and those isolated from plants may have a considerable potential for application in agriculture as a support culture for plant growth.

Methods: *P. agglomerans* DBM 3797 was cultured under aerobic and anaerobic conditions, its metabolites were analyzed by HPLC and it was tested for plant growth promotion abilities, such as phosphate solubilization, siderophore and indol-3-acetic acid productions. In addition, genomic DNA was extracted, sequenced and *de novo* assembly was performed. Further, genome annotation, pan-genome analysis and selected genome analyses, such as CRISPR arrays detection, antibiotic resistance and secondary metabolite genes identification were carried out.

Results and discussion: The typical appearance characteristics of the strain include the formation of symplasmata in submerged liquid culture and the formation of pale yellow colonies on agar. The genetic information of the strain (in total 4.8 Mb) is divided between a chromosome and two plasmids. The strain lacks any CRISPR-Cas system but is equipped with four restriction-modification systems. The phenotypic analysis focused on growth under both aerobic and anaerobic conditions, as well as traits associated with plant growth promotion. At both levels (genomic and phenotypic), the production of siderophores, indoleacetic acid-derived growth promoters, gluconic acid, and enzyme activities related to the degradation of complex organic compounds were found. Extracellular gluconic acid production under aerobic conditions (up to 8 g/l) is probably the result of glucose oxidation by the membrane-bound pyrroloquinoline quinone-dependent enzyme glucose dehydrogenase. The strain has a number of properties potentially beneficial to the hop plant and its closest relatives include the strains also isolated from the aerial parts of plants, yet its safety profile needs to be addressed in follow-up research.

KEYWORDS

***Pantoea agglomerans*, hops endophyte, plant growth promotion, genome characterization, gluconic acid**

1 Introduction

The Czech Republic is famous for its Pilsner beer, in which hops (*Humulus lupulus* L.) is irreplaceable feedstock. Hops (*Humulus lupulus* L.) is a perennial, dioecious, climbing plant belonging to the *Cannabaceae* family and order of *Rosales* (Zhang et al., 2011). Hop cones of the female plant contain in lupulin glands a lot of secondary metabolites which are mostly used in beer production. Hop resins, essential oils and their transformation products impart beer its typical bitter taste and hoppy aroma (Jaskula et al., 2008). A number of substances contained in hops have at the same time many biologically active effects. 8-Prenylnaringenin is known to be the most potent phytoestrogen to date (Milligan et al., 2000). Beta acids are characterized by strong antimicrobial effects against some groups of bacteria (Sleha et al., 2021; Fahle et al., 2022). Xanthohumol from the group of prenylated flavonoids has anticarcinogenic effects against certain types of cancer (Miranda et al., 1999).

Till now, hop plant research has focused on topics other than the natural colonization of the hop plant by endo- and epiphytic bacteria, with only a few exceptions (Goryluk-Salmonovicz et al., 2016; Allen et al., 2019; Micci et al., 2022). On the contrary, it was rather assumed that hop would not be colonized by bacteria because many of its metabolites have antimicrobial properties (for a review see, Bocquet et al., 2018). The considerable resistance of hops to bacteria is also evidenced by the fact that bacterial diseases of hops are rare compared to viruses or diseases caused by fungi. Nevertheless, some bacteria have already been isolated from hops—bacteria of the genus *Streptomyces* from the rhizosphere of hops (Koçak, 2019), *Pseudomonas stutzeri* and *Pseudomonas fluorescens* from hop cones (Sevigny et al., 2019) and *Pantoea agglomerans* from hop cones (Sevigny et al., 2019) and from dried hop pellets (Kolek et al., 2021).

Various *Pantoea* species and strains have been isolated as free-living bacteria from different habitats or from different hosts, having loose or tighter relationships to the host, i.e., many of them being plant epiphytes/endophytes, sometimes plant pathogens, others being insect symbionts or facultative human pathogens (Walterson and Stavrinides, 2015). Specifically, different strains of *P. agglomerans* were isolated from different plants (Walterson and Stavrinides, 2015) or were found as clinical isolates causing various health problems (Soutar and Stavrinides, 2019). In the same time, other *P. agglomerans* strains had beneficial properties in medicine (such as macrophage activation or to combat *Plasmodium* parasites) while yet other strains can become biocontrol agents or mediate improved plant nutrition, which might be very useful for future sustainable agricultural practice (Dutkiewicz et al., 2016). To identify differences between plant and clinical isolates through their genomes is difficult (Rezzonico et al., 2009) and is complicated by changing taxonomy and frequent misidentification of *P. agglomerans* clinical isolates (Rezzonico et al., 2009; Soutar and Stavrinides, 2019). Regarding taxonomy, into the species name *P. agglomerans* were transferred all older species originally called *Enterobacter agglomerans* or *Erwinia herbicola* and these species names are used as synonyms now. However, older isolates of *E. agglomerans* or *E. herbicola* frequently differ from *P. agglomerans* (Soutar and Stavrinides, 2019).

Despite a number of published materials, little attention has been devoted to the fact that *P. agglomerans,* as a facultative anaerobic bacterium, behaves very differently under different conditions and can switch between different metabolic pathways. In particular its anaerobic metabolism has been neglected, but can harbor surprises. *P. agglomerans* DBM 3696 was identified as the probable causative agent of inflation (production of CO_2) in bags filled with dried hop pellets stored in a modified atmosphere (Kolek et al., 2021). This study aims to demonstrate the versatile metabolism of *P. agglomerans* DBM 3697 isolated from fresh green hop cones in the Steknik hopyards (Czech Republic) along with identification of significant metabolites, as well as the complete genome and its comprehensive analysis, stressing potentially beneficial properties that may be used in agriculture (e.g., phosphate solubilisation, siderophore and auxin (indol-3-acetic acid and its derivatives) productions and others).

Currently, about 139 *P. agglomerans* genome reports can be found in the NCBI GenBank/RefSeq database, but in only 31 cases, complete genome sequences have been published.[1] Regarding plant associated *P. agglomerans,* not showing pathogenesis, comprehensive genome analyses were performed for only five strains shown in Table 1. The complete available genomic dataset of different *P. agglomerans* strains, although it may seem extensive at first glance, is in fact insufficient for differential analysis of the genomes to find significant differences between beneficial and pathogenic strains. To be able to do so, it is necessary to expand this dataset to include strains that do not show pathogenesis to plants or humans, as well as clinical isolates or strains associated with plant pathogenesis.

2 Material and methods

2.1 The strain isolation and its storage

The strain *Pantoea agglomerans* DBM 3797, deposited in the culture collection of the Department of Biochemistry and Microbiology (DBM) of the UCT Prague, was stored at −80°C. The strain was isolated from fresh hop cones grown in Steknik hopyards in the Czech Republic (altitude 192 m, latitude and longitude: 50.3166292 N 13.6102039 E). The plant material was collected under aseptic conditions in a sterile plastic bag, surface sterilization with ethanol was performed in the laboratory, the material was ground and suspended in sterile physiological solution (0.9% NaCl). The solid particles were then filtered under aseptic

Abbreviations: DBM, Department of Biochemistry and Microbiology of the University of Chemistry and Technology in Prague; EMP, Embden-Meyer-Parnas; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LB, Lysogeny Broth; OD, optical density; PGM, Pantoea glucose medium; PGP, plant growth promoting; PP, pentose phosphate; PQQ, pyrroloquinoline quinone; PS, phosphate solubilization; PTS, phosphotransferase system; rNDP, ribonucleoside diphosphate reductase; RNR, ribonucleotide reductase.

1 NIH, National Center for Biotechnology Information (2024). Available online at: https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=549&annotated_only=true&refseq_annotation=true&genbank_annotation=true&assembly_level=3%3A3.

TABLE 1 Comprehensive genome analyses of plant associated non-pathogenic *P. agglomerans*.

Strain	Plant association	Genome information	Specific features	Reference
P5	Soil sample	5.04 Mb Scaffold assembly	Potential biofertilizer	Shariati et al., 2017
C1	Isolated from the phyllosphere of lettuce (*Lactuca sativa*)	4.85 Mb 21 contigs	Plant growth-promoting (PGP) bacterium in heavy metal polluted soils	Luziatelli et al., 2020a
ANP8	Isolated from root nodules of alfalfa (*Medicago sativa*) grown in saline soil	5.03 Mb Scaffold assembly	PGP activities in saline soil	Noori et al., 2021
CPHN2	Isolated from chickpea (*Cicer arietinum*) non-rhizobial nodule	4.8 Mb (chromosome and 2 plasmids) 32 contigs	Potential biofertilizer	Kumar et al., 2022
DAPP-PG 734	Endophytic bacterium, isolated from knots (tumors) of olive tree (*Olea europaea*)	5.4 Mb (chromosome and 4 plasmids) Five contigs	Potential biocontrol activity	Sulja et al., 2022
DBM 3797	Isolated from fresh green hop (*Humulus lupulus*) cones	4.8 Mb (chromosome and two plasmids) Complete genome	PGP activities	This study

conditions through sterile folded filter paper and the filtrate was diluted 10×, 100× and 1,000×. From each dilution, 0.1 ml was inoculated onto the surface of the solidified LB medium. The plates were incubated for 24 or 48 h at 30°C. From the initial growth, the culture was plated several times on the surface of the agar medium and individual colonies were isolated.

2.2 Culture conditions

All chemicals for preparation of culture media, as well as for microbiological assays of plant growth promoting activities, were purchased from Merck if not stated otherwise. The strain was cultured in Lysogeny Broth (LB) culture medium containing (g/l): tryptone 10, yeast extract 5 and NaCl 5 or in Pantoea glucose medium (PGM) containing (g/l): glucose 10 or 20, $MgSO_4.7H_2O$ 0.4, NaCl 1, $CaCl_2.2H_2O$ 0.2, NH_4NO_3 1.5; yeast extract 0.2, KCl 0.2, peptone 0.5. The ability to utilize different carbon sources was tested in PGM, where glucose was changed for xylose, cellulose (Avicel) or lignin, always at a concentration of 10 g/l. For bioreactor culture, the glucose concentration was 20 g/l. In some experiments, LB culture medium was supplemented with glucose at a concentration of 10 g/l. For growth in Petri dishes, culture medium was supplemented with 20 g/l of agar. For indole-3-acetic acid production, tryptophan was added to the culture medium at a concentration of 5 g/l. Culture experiments were performed at 30°C for 24–48 h. Each inoculum for culture experiments was prepared by overnight growth in LB liquid medium.

Cultivation experiments were run in Erlenmeyer shake flasks on a rotary shaker (150 rpm), in a 1 l bioreactor (Infors HT), both aerobically and anaerobically, in a thermostat (the case of growth on solidified medium in Petri dishes) or in an anaerobic chamber (Concept 400, UK). For bioreactor experiments, PGM culture medium with 20 g/l glucose was used. The working volume of the 1 l bioreactor was 700 ml (630 ml of fresh culture medium and 70 ml of inoculum) and pH monitoring were used. In aerobic culture, the filtered air rate was 1 VVM and oxygen saturation was measured using an oxygen electrode. Details of anaerobic bioreactor culture were described previously by Sedlar et al. (2021).

2.3 Analyses

Growth was monitored as an optical density (OD) at 600 nm using a spectrophotometer (Agilent Cary 60 UV-VIS) against the respective medium without inoculation as a blank. Microscopic control of the culture was performed using phase contrast microscopy (Olympus BX51; Olympus).

The concentration of substrate (glucose) and metabolites (ethanol, lactic, acetic and gluconic acids) were determined by HPLC (Agilent Series 1200 HPLC; Agilent) with refractive index detection. The parameters of the HPLC analysis were as follows: injection sample volume of 20 µl, 5 mM H_2SO_4 as a mobile phase, a flow rate of 1 ml/min, IEX H^+ polymer column (Watrex) and a column temperature of 60°C.

Statistical analysis of different growth conditions was performed in R (v4.3.1). Data normality was checked with Shapiro-Wilk test (p-value < 0.05) and homogeneity of variance was verified using Bartlett's test (p-value < 0.05). One-way ANOVA with *post-hoc* Tukey test was performed at p-adjusted value < 0.05 to identify statistically significant changes under different cultivation conditions.

2.4 DNA extraction and sequencing

For short-read sequencing, genomic DNA was extracted and purified using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MI, USA) following the manufacturer's protocols. The purity of the DNA was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), while the concentration was determined using the Qubit 3.0 (Thermo Scientific, Wilmington, DE, USA). DNA library construction was carried out using the KAPA HyperPlus kit,

following the standard protocol. Subsequently, sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v2 (500 cycles).

For long-read sequencing, high molecular weight genomic DNA was extracted using the MagAttract HMW DNAKit (Qiagene, Venlo. NL). The purity of the extracted DNA was assessed with the NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), while the concentration was determined using the Qubit 3.0 (Thermo Scientific, Wilmington, DE, USA). The DNA length was confirmed using the Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Ligation sequencing 1D Kit (Oxford Nanopore Technologies, Oxford, UK) was used for library preparation, and sequenced on the MinION platform (Oxford Nanopore Technologies) with the R9.4.1 flowcell.

2.5 Genome assembly

Long Oxford Nanopore Technologies (ONT) reads were basecalled with Guppy v3.4.4 and used for the initial *de novo* assembly performed with Flye v2.8.1. The assembly was polished with Racon v1.4.13 (Vaser et al., 2017) and Medaka v1.2.5 using ONT reads quality checked with MinIONQC (Lanfear et al., 2019). Auxiliary PAF files were generated using minimap2 (Li, 2018). Short Illumina paired reads were quality trimmed with Trimomatic v1.36 (Bolger et al., 2014), checked with FastQC v0.11.5 and MultiQC v1.7 (Ewels et al., 2016), and used for additional rounds of polishing with Pilon v1.24 (Walker et al., 2014). For that purpose, short reads were mapped onto ONT assembly with BWA v07.17 (Li and Durbin, 2009) and auxiliary BAM files were processed with SAMtools (Li et al., 2009). Finally, the resulting chromosomal sequence was rearranged according to the origin of replication (*oriC*) to *dnaA,* being the first gene on the sense strand, using the Ori-finder (Luo et al., 2019) and both plasmid sequences were rearranged in a similar manner to the *repB* gene coding for plasmid replication initiator, being the first gene on the sense strand, using manual BLAST searches (Altschul et al., 1990).

2.6 Genome annotation and analysis

Genome annotation was performed by NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The functional annotation of protein coding genes was extended by classification into categories of clusters of orthologous groups (COG). Overall three sources of COG categories were used, namely eggNOG-mapper (Cantalapiedra et al., 2021) (v2.1.9), Operon-mapper (Taboada et al., 2018) and Batch CD-Search (Marchler-Bauer and Bryant, 2004) tools. Results were further processed by COGtools (v1.0.0) (https://github.com/xpolak37/COGtools) to merge them and create a final improved COG annotation. Assigned COG categories were visualized as circular plots by DNAplotter (Carver et al., 2009), which is a part of the Artemis (Carver et al., 2012) (v2.18.0) software. Selected pathogenic and non-pathogenic chromosomal sequences were compared and visualized as a circular graph in BRIG (v0.95) software (Alikhan et al., 2011). Pan-genome analysis was performed using BPGA v1.3 (Chaudhari et al., 2016), with amino acid sequences clustered using USEARCH (Edgar, 2010), with an identity cut-off of 90%. In total, 139 genomes of *P. agglomerans* were obtained from the NCBI RefSeq database (30th October 2023) (O'Leary et al., 2016) to define the core genome and to perform a phylogenomic analysis, i.e., concatenated sequences of core genes were aligned with MUSCLE and resulting multiple sequence alignment was used to reconstruct phylogeny with Neighbor-Joining algorithm using Kimura distance implemented in BPGA.

The genome was searched for clustered regularly interspaced short palindromic repeat (CRISPR) arrays using the CRISPRDetect (Biswas et al., 2016) (v2.4) tool and *cas* genes were searched in the genome manually. Components of restriction-modification (RM) systems were identified using REBASE (v307) database (Roberts et al., 2023). Prophage DNA was searched with the online version of PHASTER (Arndt et al., 2016). Antibiotic-resistant genes search was performed using Resistance Gene Identifier (RGI) 6.0.0 included in the Comprehensive Antibiotic Resistance Database (CARD) 3.2.5 (Alcock et al., 2020) by submitting protein sequences of CDSs. Virulence factors were searched using online version of VFAnalyzer against the virulence factor database (VFDB) (Liu et al., 2019) with default parameters and using *Klebsiella pneumoniae* as the closest annotated reference for *P. aglomerans*. Homologs of genes involved in biosynthetic pathways, putatively contributing to plant growth promotion and other activities were identified with tBLASTn, with the use of target protein sequences from closely related species. The length of initial seeds was set to 5 and BLOSUM62 matrix was used for scoring the alignments while gap introduction and extension was set to 11 and 1, respectively. Finally, identification of secondary metabolite biosynthesis gene clusters was performed with antiSMASH v7.1.0 (Blin et al., 2023) through its web service using relaxed detection strictness parameter.

2.7 Plant growth promoting activities

Screening of PGP activities was performed by established microbiological assays combined with spectrophotometric or visual detection and frequently (if not stated otherwise) at a semi-quantitative level (low, medium, or high).

Siderophore production was tested on blue agar chrome azurol S medium containing chrome azurol S and hexadecyltrimethylammonium bromide as indicators. Development of a yellowish orange halo around the colonies was taken as indicative of siderophore production; for details see Schmidt et al. (2018).

Phosphate solubilization was detected as a clear zone, i.e., the ability to solubilize calcium phosphate using Pikovskaya medium see Schmidt et al. (2018) or was tested in liquid NBRIP medium where the concentration of phosphate was determined spectrophotometrically by the ammonium molybdate-ascorbic acid method (Stranska et al., 2021).

Nitrogen fixation ability was tested in NFGM medium and evaluated spectrophotometrically; details are presented in Stranska et al. (2021).

Amylase, lipase, pectinase, protease/peptidase, and cellulase production were tested for in the appropriate solidified culture

medium (Hawar, 2022) and evaluated as a halo or colored zone around a colony.

Ammonium release was detected after 24 h growth in LB medium by the Quantofix rapid test following instructions of the producer (Quantofix).

Indole-3-acetic acid (IAA) or IAA-like compound production was tested by Salkowski reagent (0.01 M $FeCl_3$ in 35% $HClO_4$) in LB culture medium supplemented with tryptophan after 48 h growth on a rotary shaker; for details of the procedure see Gilbert et al. (2018).

Indole production/release was tested by reaction with Kovacs reagent (Merck) in LB culture medium after 24 h growth on a rotary shaker.

3 Results

3.1 Genome and pan-genome

The genome of *P. agglomerans* DBM 3797 comprises a circular chromosome (size 4,089 kb) and two circular plasmids (pPA_DBM3797_1 size 555 kb and pPA_DBM3797_2 size 182 kb) assembled using both long reads and short reads in a hybrid approach with an overall coverage of 584× and deposited at the DDBJ/EMBL/GenBank under accession numbers CP086133.1, CP086134.1, and CP086135.1, respectively. The overall genome length is 4,827,556 bp and contains 4,486 open reading frames (ORFs). While 4,328 ORFs present protein-coding sequences (CDSs), 49 genes had corrupted ORFs and formed pseudogenes. The remaining loci corresponded to RNA coding genes. Statistics for chromosome and both plasmids are summarized in Table 2. While most genes putatively corresponding to phenotypic traits were found on the chromosome, some of them were located on the large pPA_DBM3797_1 plasmid.

Functional annotation of the genome was done by classifying protein coding genes and pseudogenes into 26 categories of clusters of orthologous genes (COG), see Figure 1. For the chromosomal sequence, 3,280 genes were assigned a COG while 419 genes (11.33%) remained unannotated. Three most abundant categories were categories: E (Amino acid transport and metabolism) with 323 genes (8.73%), G (Carbohydrate transport and metabolism) with 321 genes (8.68%), and M (Cell wall/membrane/envelope biogenesis) with 268 genes (7.25%). For plasmid pPA_DBM3797_1 sequence, 66 genes (12.48%) remained unannotated and from 463 remaining genes, 78 genes (14.74%) were assigned category K (Transcription) and 69 genes (13.04%) category G (Carbohydrate transport and metabolism), forming the two most abundant groups. Moreover, 130 genes found on plasmid pPA_DBM3797_2 were assigned a COG while the most frequent was category T (Signal transduction mechanism) with 20 genes (13.42 %). Other abundant groups were formed by genes assigned to K (Transcription) and V (Defense mechanisms), both containing 15 genes (10.07%). Nineteen genes (12.75%) remained unannotated. For a complete summary of COG statistics see Supplementary Table 1. Additionally, seven secondary metabolite biosynthetic gene clusters were found. While five regions corresponding to redox-cofactor (2,192,097–2,214,263), arylpolyene and homoserine lactone (hserlactone) (2,614,136–2,673,970), thiopeptide (2,739,933–2,766,189), hserlactone (3,555,694–3,576,332), and NRP (non-ribosomal peptide)-metallophore and NRPS (non-ribosomal peptide synthase) (3,660,174–3,713,865) were located on the chromosome, remaining two regions corresponding to NI-siderophore (165,106–195,460) and terpene (376,789–400,350) were located on the pPA_DBM3797_1 plasmid (Supplementary Figure 1).

The *P. agglomerans* genome was missing any clustered regularly interspaced short palindromic repeat (CRISPR) arrays and similarly, no *cas* genes are present. Furthermore, we found four restriction-modification (R-M) systems, one was of type I and the remaining three were of type II. A type I R-M system consisted of one restriction enzyme Pag3797ORF16840P, two methyltransferases M1.Pag3797ORF16840P and M2.Pag3797ORF16840P, and one specificity subunit S.Pag3797ORF16840P. In all three type II systems, we found methyltransferases: M.Pag3797DamP, M.Pag3797ORF3130P and M.Pag3797DcmP, while the last enzyme was also coupled with nicking enzyme V.Pag3797DcmP. Complete results for R-M systems can be found in Supplementary Table 2. Only a single intact prophage of length 41.8 kbp corresponding to phage PHAGE_Erwini_ENT90_NC_019932 was found on the chromosome, within region 648492-690301. The whole region contained 57 proteins in total while 53 of these genes corresponded to phage DNA.

Last but not least, the genome was searched for antibiotic resistance and virulence genes. In total, 12 strict hits were found

TABLE 2 Genome features of *P. agglomerans* DBM 3797.

Feature	Chromosome	pPA_DBM3797_1	pPA_DBM3797_2
Length (bp)	4 089 578	555 522	182 426
GC content (%)	55.5	52.5	53.0
ORFs	3,808	529	149
CDSs	3,673	508	147
Pseudogenes	26	21	2
rRNA genes (5S, 16S, 23S)	8, 7, 7	0, 0, 0	0, 0, 0
tRNAs	77	0	0
ncRNAs	10	0	0

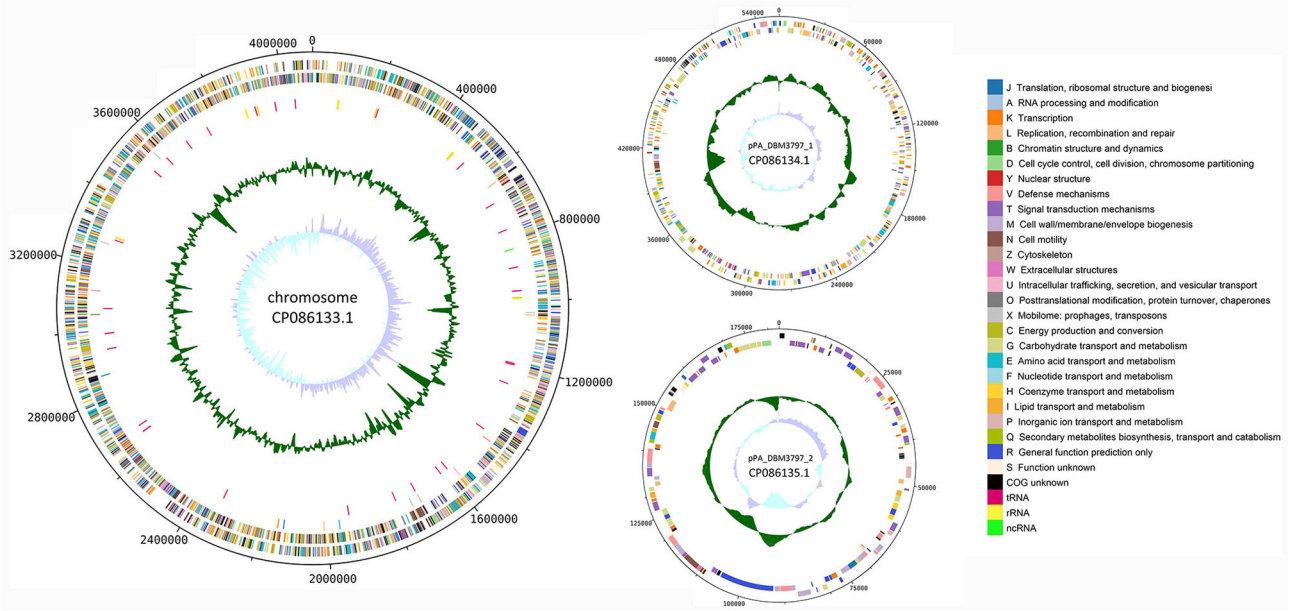

FIGURE 1
A circular map of the chromosome **(on the left)** and both plasmid **(on the right)** sequences of *P. agglomerans* DBM 3797. From outside to center: CDS on the forward strand (color-coded by COG categories), CDS on the reverse strand (color-coded by COG categories), pseudogenes (color-coded by COG categories), RNA genes (tRNA, rRNA, ncRNA), GC content and GC skew.

in the Comprehensive Antibiotic Resistance Database. While 11 genes were localized on chromosome, the remaining gene was found on plasmid pPA_DBM3797_2, see Supplementary Table 3. Six of these genes corresponded to antibiotic efflux resistance mechanisms, five were predicted to be responsible for antibiotic target alternation and one for antibiotic inactivation. The presence of these genes was confirmed by searching for virulence factors in general using the Virulence Factor Database. The presence of other virulence factors remained inconclusive as only partial hits to other secretion system or endotoxin genes were detected. The only complete system corresponded to gene machinery responsible for flagella construction, however, the cell motility is not necessarily connected to virulence.

The chromosomal sequence of *P. agglomerans* DBM 3797 was compared with chromosomal sequences of selected pathogenic and non-pathogenic strains downloaded from GenBank database under further mentioned accession numbers. These included the only pathogenic available strain isolated from clinical, FDAARGOS 1447 (CP077366.1); a plant pathogenic strain, BH6c (CP134744.1); and three non-pathogenic strains isolated from the same plant part (above-ground part) as DBM 3797, namely DAPP-PG734 (OW970315.1), CPHN 2(CP098414.1), and CFSAN047154 (CP034474.1). The results of a comparative analysis revealed no significant differences among the chromosomal sequences. All analyzed sequences were aligned to the reference strain DBM 3797 with 100% identity along almost the entire length of the sequence (see Supplementary Figure 2).

The pan-genome analysis showed that all currently available genomes of *P. agglomerans* strains with successful taxonomy check shared 2,399 genes that formed the core genome of the species. Phylogenomic tree reconstructed using concatenated sequences of all core genes showed that *P. agglomerans* DBM 3797 presented a well-distinguished strain with strains AB378 and CFBP8784 being the closest relatives, see Figure 2. The complete list of the strains included into the Figure 2 is shown in Supplementary Table 4.

3.2 Growth, metabolite formation, and putative corresponding genes

P. agglomerans DBM 3797 was grown under aerobic and anaerobic conditions in liquid medium. Ability to grow in the presence/absence and limitation of oxygen requires security of basic life functions under both conditions, such as ability to synthesize deoxyribonucleotides by ribonucleotide reductases (RNR). RNRs mediate the reduction of nucleotides differently under aerobic/anaerobic conditions and for this, different enzymes are required. The corresponding RNR genes located on the chromosome are shown in Table 3. Under aerobic conditions, the strain preferred LB medium, which did not contain saccharides. Young cells (5 h after inoculation) were highly motile while in older LB-medium, in the grown population (after 24 h), symplasmata formation was observed (Supplementary Figure 3). Putative genes responsible for motility and symplasmata formation (biofilm like structure) were found on the chromosome and are shown in Supplementary Table 5. It was also tested whether symplasmata formation might be initiated by indole release during tryptophan degradation (tryptophan presence was assumed in LB medium) by the reaction of culture medium supernatant with Kovacs reagent, but the reaction was negative. In addition, the gene for tryptophanase was not found on the chromosome, nor on the

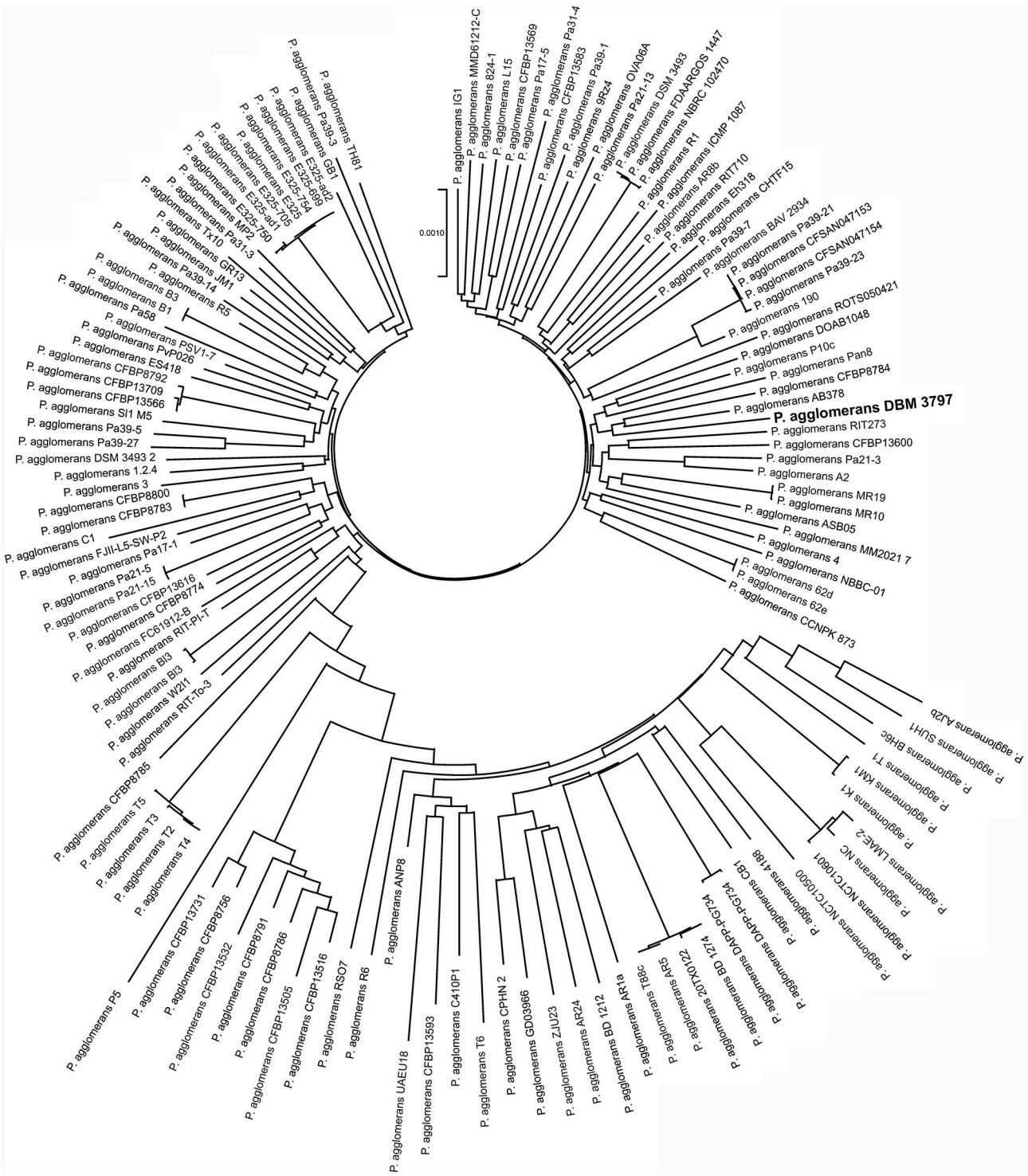

FIGURE 2
Phylogenomic analysis based on concatenated sequences of 2,399 core genes of 139 genomes of *P. agglomerans* strains. The tree was reconstructed using the Neighbor-Joining method using the Bacterial Pan Genome Analysis tool (BPGA).

plasmids. An interesting feature of growth in LB medium was alkalization of the culture medium (up to pH 8.7, see Table 4), which was caused by the release of ammonium ions from amino acids, serving as a carbon source in the medium. Ammonium ion concentration was about 100–200 mg/l. Aerobically, the culture was able to utilize glucose, xylose, cellulose (Avicel) and lignin, however compared to LB medium growth, the cells were shorter, the amount of biomass formed in 24 h was about 10 times lower and no symplasmata were observed. Anaerobic growth required saccharides for a fermentative way of obtaining

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TABLE 3 Ribonucleotide reductase [ribonucleoside diphosphate reductases (rNDP)] genes.

Gene locus	Gene product annotation	Gene abbreviation
Ribonucleoside reductase class I (aerobic)		
LKW31_04835	Class Ib ribonucleoside-diphosphate reductase subunit beta	*nrdF*
LKW31_04840	Class Ib ribonucleoside-diphosphate reductase subunit alpha	*nrdE*
LKW31_04845	Class Ib ribonucleoside-diphosphate reductase assembly flavoprotein NrdI	*nrdI*
LKW31_06395	Class I ribonucleotide reductase maintenance protein YfaE	*yfaE*
LKW31_06400	Class Ia ribonucleoside-diphosphate reductase subunit beta	*nrdB*
LKW31_06405	Class Ia ribonucleoside-diphosphate reductase subunit alpha	*nrdA*
Ribonucleoside reductase (anaerobic)		
LKW31_17070	Anaerobic ribonucleoside-triphosphate reductase	*nrdD*
LKW31_17075	Anaerobic ribonucleoside-triphosphate reductase-activating protein	*nrdG*

energy and therefore was not possible in simple LB medium not containing glucose. On solidified LB medium under aerobic conditions, the strain formed round, convex, slimy looking, cream to yellowish colored colonies (see Supplementary Figure 4). Genes for carotenoid pigment production, giving the colony a yellowish color, were found on plasmid pPA_DBM3797_1, see Supplementary Table 5 and Supplementary Figure 1 (terpene).

Under all conditions, acids were formed as the main primary metabolites, together with a small amount of ethanol. Acid formation resulted in a pH drop that caused growth to slow down and finally stop. While under anaerobic conditions, the main metabolites were acetic and lactic acids and ethanol, whereas under aerobic conditions, most of the glucose was oxidized to gluconic acid, plus the formation of lactic and acetic acids and ethanol. The concentration of lactic acid and the cell dry weight have rather significantly changed based on cultivation medium than based on aerobic/anaerobic conditions. However, statistically significant change based on aerobic/anaerobic conditions was observed for acetic acid and gluconic acid (with exception for LB aerobic cultivation condition). Ethanol level was similar under all conditions with no statistically significant change. Comparison of acid and ethanol production under different culture conditions is shown in Table 4 while the candidate genes coding for pyruvate processing into lactic and acetic acids and ethanol are shown in Table 5.

Cultivations under different oxygen availability were also compared during bioreactor cultivations using PGM with 20 g/l of glucose (Supplementary Figure 5). Under aerobic conditions in a bioreactor, glucose consumption was double that under anaerobic conditions, but a substantial fraction of glucose was oxidized to gluconic acid. Oxygen limitation was observed during aerobic bioreactor culture, demonstrated as zero oxygen saturation from the 4th to the 14th hour of cultivation. The extracellular concentration of gluconic acid achieved was about 6 g/l in the bioreactor experiment using PGM and up to 8 g/l in shake flask experiments where LB medium supplemented with glucose (10 g/l) was used (see Table 5). A scheme demonstrating the putative gluconic acid metabolic pathways was created, see Figure 3, and respective candidate genes are shown in Table 6. While it seems that gluconic acid production is mostly mediated by membrane bound enzymes and is extracellular, gluconate can be transported into a bacterial cell by a specific gluconate transporter and this transport might be coupled with phosphorylation. The resulting 6-phospho-gluconate may be processed to metabolites entering either the Entner-Doudoroff or Pentose Phosphate pathways, see Figure 3.

3.3 Plant growth promotion

Plant growth promoting activities were tested in a series of traditional microbiological assays and the candidate genes for all PGP activities are shown in Supplementary Table 5. There were confirmed high proteolytic/peptidase and cellulase activities, medium siderophore and IAA related compounds productions, weak amylolytic, lipolytic and pectinase activities. The ability to form indole acetic acid (IAA) or IAA-like compounds was tested in culture medium supplemented with the precursor compound, tryptophan and a positive reaction with Salkowski reagent was obtained. As the color was distinct compared to standard (IAA) as well as its retention time in UHPLC analysis (not shown), it is probable that not directly IAA, but a similar compound is formed. The most well-known gene of the IAA pathway, indolepyruvate decarboxylase, *ipdC*, was found in the genome (Supplementary Table 5). Further, symplasmata (biofilm like structure), carotenoid pigment formation and the ability to release ammonium mentioned above, can be considered PGP activities too. The ability to degrade ethylene was not tested, however the putative gene for 1-aminocyclopropane-1-carboxylate (ACC) deaminase was found on the chromosome. Phosphate solubilisation (PS) was tested in different types of tests but was not confirmed even if the genes for phosphonate metabolism and phosphate transporters were found in the genome (Supplementary Table 5) and gluconic acid formation was demonstrated.

4 Discussion

The *P. agglomerans* DBM 3797 strain isolated from fresh hop has a somewhat different phenotype from the similar strain *P. agglomerans* DBM 3796 isolated from dried hop (Kolek et al., 2021) and differed mainly in the low production of CO_2 associated

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TABLE 4 Comparison of growth, acid and ethanol formation under aerobic or anaerobic conditions.

Culture conditions	PGM aerobic	PGM anaerobic	LB with glucose, aerobic	LB with glucose, anaerobic	LB aerobic
Lactic acid (g/l)	0.76 ± 0.12^{b}	0.67 ± 0.11^{b}	2.23 ± 0.17^{c}	1.88 ± 0.05^{c}	0.30 ± 0.02^{a}
Acetic acid (g/l)	0.07 ± 0.01^{a}	0.12 ± 0.01^{b}	0.08 ± 0.01^{a}	0.15 ± 0.01^{b}	ND
Gluconic acid (g/l)	6.14 ± 0.12^{b}	0.21 ± 0.02^{a}	8.02 ± 0.20^{c}	0.42 ± 0.07^{a}	0.44 ± 0.12^{a}
Ethanol (g/l)	0.35 ± 0.03^{a}	0.27 ± 0.03^{a}	0.34 ± 0.02^{a}	0.35 ± 0.04^{a}	ND
Cell dry weight (g/l)	1.10 ± 0.15^{a}	0.90 ± 0.10^{a}	2.80 ± 0.10^{b}	2.50 ± 0.05^{b}	3.70 ± 0.15^{c}
pH	4.3 ± 0.1^{b}	4.9 ± 0.1^{c}	3.3 ± 0.1^{a}	6.4 ± 0.1^{d}	8.7 ± 0.1^{e}

Glucose concentration in PGM and LB media with glucose was 10 g/l, culture experiments were performed in Erlenmeyer flasks in triplicate on a rotary shaker (aerobic condition) or in an anaerobic chamber (anaerobic conditions) for 48 h, and the pH of culture medium before inoculation was 6.8. ND, not detected; values labeled with identical letters are not significantly different at p-adjusted value < 0.05.

with low production of ethanol and acetic acid. The strain has two plasmids, whose circularity was proven during *de novo* assembly that produced circular contigs. Moreover, both plasmids contained the *repB* gene coding for plasmid replication initiator, suggesting that both plasmids formed integral parts of the *P. agglomerans* genome rather than foreign DNA. Additionally, no intact prophage sequences were found on plasmids. The first plasmid pPA_DBM3797_1, of size 555 kb and harboring genes for carotenoid biosynthesis and siderophores (Supplementary Table 5), as well as thiamine biosynthesis (not shown) genes; these seem to meet the criteria for a large universal *Pantoea* plasmid (De Maayer et al., 2012). Growth under both aerobic and anaerobic conditions correlates with the possibility of synthesizing deoxyribonucleotides for DNA replication during growth by class I (aerobic) and class III (anaerobic) ribonucleotide reductases (Torrents, 2014). The second plasmid, pPA_DBM3797_2 is, according to a functional annotation, responsible for signal transduction and defense mechanisms rather than metabolism and carries one gene responsible for antibiotic resistance from pmr phosphoethanolamine transferase gene family. This gene might be involved in polymyxin resistance (Huang et al., 2018) and there is a potential risk for its spreading by horizontal gene transfer. Nevertheless, the risk assessment requires further study. Other antibiotic resistance genes are of lower risk as they are located on chromosome and in addition, a lot of them are efflux pumps genes which might be attributed to the need to resist the action of antimicrobial substances produced by the host hop plant. The antimicrobial active substances of hops include, for example, beta-acids, effective against methicillin resistant *Staphylococcus aureus* strains (Sleha et al., 2021). The absence of a native CRISPR-Cas system that can serve as a form of bacterial immunity (Sorek et al., 2013) is compensated for by the presence of numerous R-M systems. At least some of these systems are probably active, as *P. agglomerans* DBM 3797 contains only a minimum of foreign DNA, particularly only one intact prophage PHAGE_Erwini_ENT90_NC_019932. The presence of such foreign DNA is not unique for *P. agglomerans* as the very same prophage was previously identified in the genome of the strain *P. agglomerans* C1 (Luziatelli et al., 2019).

The most closely related strains, AB378 and CFBP8784, were, like strain DBM 3797, isolated from the phyllosphere of

TABLE 5 Candidate genes for metabolite formation from pyruvate.

Gene locus	Gene product annotation	Gene abbreviation
Lactic acid formation		
LKW31_13490	D-lactate dehydrogenase	
LKW31_15200	FMN-dependent L-lactate dehydrogenase LldD	*lldD*
Ethanol formation		
LKW31_09010	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	*adhE*
Acetate formation (anaerobic conditions)		
LKW31_12905	Formate transporter FocA	
LKW31_12910	Formate C-acetyltransferase	*pfl*
LKW31_12915	Pyruvate formate lyase 1-activating protein	
LKW31_06270	Phosphate acetyltransferase	*pta*
LKW31_06275	Acetate kinase	*ack*

plants, specifically from Red Topaz apple blossom (AB378) and from radish *Raphanus sativus* flower (CFBP8784). Together with other neighboring strains (Figure 2), i.e., Pan8 (isolated from *Pisum sativum* phyllosphere), P10c (isolated from apple tree), and DOAB1048 (isolated from wheat leaves) they form a group of *P. agglomerans* environmental strains isolated from above-ground plant parts and differ from strains P5, ANP8, CPHN2, and DAPP-PG744 (Table 1) isolated from plant roots or from soil.

The ability to form a biofilm is considered to be an advantage for the bacteria colonizing the plants, as the biofilm protects both the bacterial population from adverse environmental influences and the colonized plant surface. In addition, the ability to communicate between the microbial community and the plant cells is enhanced by signal amplification during biofilm formation (Seneviratne et al., 2010). Symplasmata i.e., multicellular round aggregates mimicking colonies in a liquid medium, probably gave the original name to the species "agglomerans" (Tecon and Leveau, 2016) and were described in detail e.g., in the rice epiphyte *P. agglomerans* YS19 (Yu et al., 2016; Zheng et al., 2019). Although

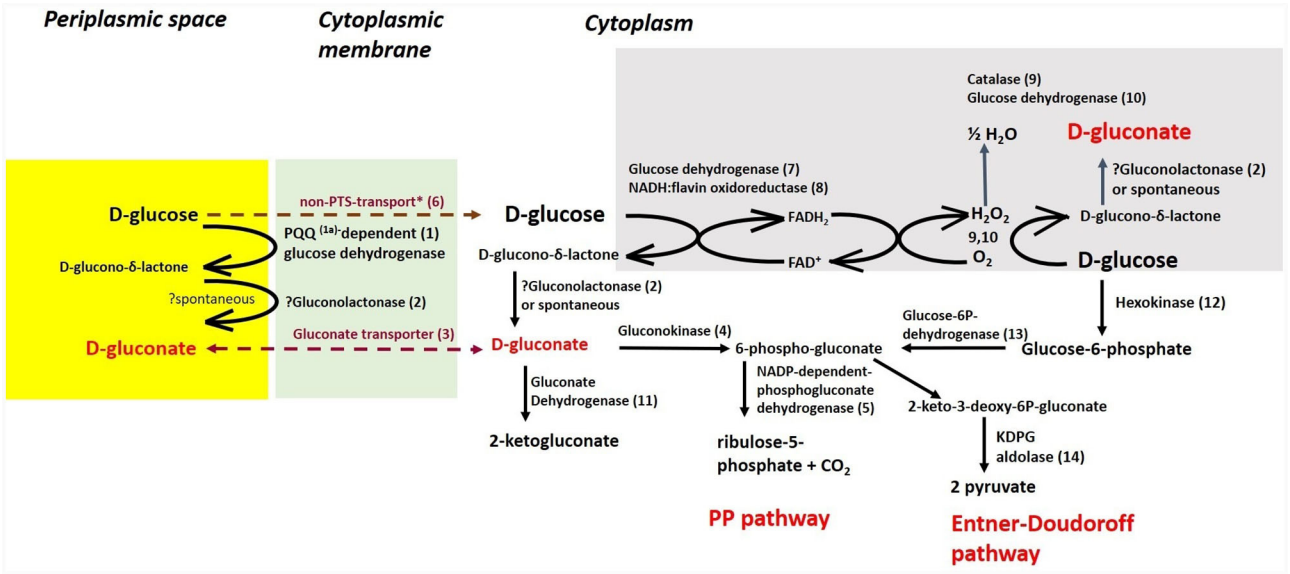

FIGURE 3
Putative pathways associated with gluconic acid production and its processing *in P. agglomerans* DBM 3797. The candidate genes for enzyme activities (1–14), as well as biosynthesis of pyrroloquinoline quinone (PQQ) (1a) are shown in Table 6. While the yellow highlighted part, which results in formation of extracellular gluconic acid, was confirmed by HPLC analysis, other proposed pathways require further confirmation. The cytoplasmic membrane is highlighted in green, intracellular gluconic acid production from glucose, atypical for bacteria, is highlighted in gray. PP stands for pentose phosphate. *Stands for supposed glucose transport by passive diffusion.

these formations were firstly described by M. W. Beijerinck in 1888 (Tecon and Leveau, 2016), it is still not completely clear why they are formed. They provide protection to the bacteria, comparable to biofilm formation, and are considered to be an advantage for colonization of rice roots (Achouak et al., 1994). Symplasmata formation seems to be initiated by culture conditions but different reports differ in descriptions of which factor is decisive. While *Pantoea eucalypti* best formed symplasmata in glucose medium not in LB medium, and at a pH higher than 7.6 (Tecon and Leveau, 2016), our strain *P. agglomerans* DBM 3797 formed them only in LB medium at pH 8.5. Our findings are in accordance with those for strain YS19 (Jiang et al., 2015; Yu et al., 2016; Zheng et al., 2019) and nitrogen-fixing *E. agglomerans* NO30 (Achouak et al., 1994). Symplasmata of strain YS19 were probably regulated by indole (Yu et al., 2016) and it was hypothesized that indole originated from tryptophan degradation and was considered by the *P. agglomerans* indolenon-producing strain as a marker of starvation (Jia et al., 2017). In addition, in the same strain YS19, an acyl-homoserine lactone quorum sensing system was also involved in symplasmata formation (Jiang et al., 2015). While the genes for acyl-homoserine lactone were confirmed in our strain by antiSMASH analysis, indole release was not confirmed. Symplasmata formation was also found in an anaerogenic group of *Enterobacter* clinical isolates (Gilardi and Bottone, 1971), which corresponds to the finding that genes for anaerobic metabolism were active during symplasmata formation in *P. eucalypti* aerobic culture (Tecon and Leveau, 2016) and it was deduced that there is an oxygen limitation inside these formations. The ability to cope with anaerobic conditions was proven in our strain.

The ability to utilize peptides in LB medium corresponds with the number of peptidase genes present in the genome (not shown). This type of fermentation is typical for certain fermented foods such as natto (fermented soybeans) or pidan (fermented eggs) rich in proteins and peptides (Wang and Fung, 1996) and was also described in detail for *E. coli* (Sezonov et al., 2007). Aerobic growth in LB medium is a special type of alkaline fermentation where amino acids are used as carbon sources instead of saccharides. Generally, it is supposed that amino acids are processed by oxidative deamination, generating α-keto acids and ammonia. It is believed that the key role in the process is played by glutamate dehydrogenase, but its respective candidate gene was not found in the genome. Thus, it seems more likely that metabolism of each amino acid is unique and while the ammonium cation is released or transferred to other compounds, the rest of the molecule can be transformed to pyruvate, acetyl CoA, acetoacetyl CoA and intermediates of the citric acid cycle under aerobic conditions, as described by Li et al. (2018). Part of the ammonium ion is used by the bacterium but its release is excessive which results in an increase in pH. Actually, the release of ammonium ions from available amino acids and peptides can be one of the advantageous features of the DBM 3797 strain, which might be used for controlled ammonia release if the strain was applied together with organic fertilizers.

In many aerobic Gram-negative bacteria, gluconic acid is formed by glucose dehydrogenase through D-glucono-δ-lactone in the periplasmic space (Ma et al., 2022). Further, it is expected that transport of gluconate from the periplasmic space through the outer membrane is mediated by porins. Based on knowledge gathered for *Gluconobacter oxydans* (Pronk et al., 1989), extracellular gluconic acid production is probably a result of the membrane bound PQQ-dependent glucose dehydrogenase, which was described in detail for *Pantoea ananatis* (Andreeva et al., 2011).

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TABLE 6 Candidate genes for gluconate metabolism found on the chromosome of *P. agglomerans* DBM 3797.

Gene locus*	Gene product annotation	Gene abbreviation
PQQ dependent membrane bound glucose dehydrogenase (1)		
LKW31_10030	Membrane-bound PQQ-dependent dehydrogenase, glucose/quinate/shikimate family	
LKW31_17685	Glucose/quinate/shikimate family membrane-bound PQQ-dependent dehydrogenase	
LKW31_05305	Glucose/quinate/shikimate family membrane-bound PQQ-dependent dehydrogenase	
PQQ biosynthesis (1a)		
LKW31_10225	Pyrroloquinoline quinone biosynthesis protein PqqF	*pqqF*
LKW31_10230	Pyrroloquinoline quinone biosynthesis protein PqqE	*pqqE*
LKW31_10235	Pyrroloquinoline quinone biosynthesis peptide PqqD	*pqqD*
LKW31_10240	Pyrroloquinoline-quinone synthase PqqC	*pqqC*
LKW31_10245	Pyrroloquinoline quinone biosynthesis peptide PqqB	*pqqB*
LKW31_10250	Pyrroloquinoline quinone precursor peptide PqqA	*pqqA*
Gluconolactonase (2)		
LKW31_05840	SMP-30/gluconolactonase/LRE family protein	
Gluconate transport + phosphorylation (3,4)		
LKW31_01595	Gluconate operon transcriptional repressor GntR	*gntR*
LKW31_01600	Gluconokinase	
LKW31_01605	Gluconate transporter	
6-phosphogluconate dehydrogenase (5)		
LKW31_07145	NADP-dependent phosphogluconate dehydrogenase	
Glucose non-PTS transport (6)		
LKW31_00160	Sugar ABC transporter permease	
Glucose dehydrogenase (7)/NADH oxidoreductase (8)		
LKW31_09960	Glucose 1-dehydrogenase	*gdh*
LKW31_09965	NADH:flavin oxidoreductase/NADH oxidase	
Catalase (9)/glucose dehydrogenase (10)		
LKW31_10630	Manganese catalase family protein	
LKW31_10635	Glucose 1-dehydrogenase	*gdh*
Gluconate dehydrogenase (11)		
LKW31_16920	Gluconate 2-dehydrogenase subunit 3 family protein	
LKW31_17770	Gluconate 2-dehydrogenase subunit 3 family protein	
Hexokinase (12)		
LKW31_05780	Glucokinase	*glk*
LKW31_10185	Glucokinase	
13,14		
LKW31_08485	MurR/RpiR family transcriptional regulator	
LKW31_08490	Glucose-6-phosphate dehydrogenase	
LKW31_08495	Bifunctional 4-hydroxy-2-oxoglutarate aldolase/2-dehydro-3-deoxy-phosphogluconate aldolase	
LKW31_02800	KDGP aldolase family protein	

*If there was a candidate transcriptional regulator in the vicinity of the candidate gene, it is shown too.

Candidate genes for this enzyme activity, as well as the complete *pqqABCDEF* biosynthetic operon, were found in the genome. In Gram-negative aerobic bacteria, there are frequently found other membrane bound enzymes, such as PQQ-dependent 5-keto-gluconate dehydrogenase, and flavin/heme dependent gluconate and 2-keto-gluconate dehydrogenases (Ma et al., 2022), but the

candidate genes were not found in the genome. Microbial gluconic acid production was reviewed by Ramachandran et al. (2006) and Ma et al. (2022) and it is obvious that fungi and bacteria differ in metabolic pathways for gluconic acid production. In fungi, such as in *Aspergillus niger*, its main industrial producer, gluconic acid is produced by FAD^+-dependent glucose oxidase, which is coupled with catalase (Ramachandran et al., 2006) and surprisingly, similar candidate genes coding for this option, which are atypical for bacteria, were also found in our genome (see Figure 3; Table 5). Further, it appears that the catabolic pathway of glucose in the studied strain may use parts of known metabolic pathways such as the Entner-Doudoroff, Embden-Meyer-Parnas or Pentose Phosphate pathways, which are interconnected, similar to what has been found and described for *Pseudomonas putida* KT2440 (Nikel et al., 2015).

Gluconic acid production may be associated with defense against soil protozoa such as *Vahlkampfia* sp. or *Neobodo designis* (Gómez et al., 2010), however mostly it is exploited together with other organic acids in the solubilization of inorganic phosphate. Phosphate solubilization is a significant PGP feature that facilitates plant growth by increasing its accessibility from both inorganic and organic phosphate-containing compounds and complexes (Rawat et al., 2021). Typical phosphate solubilizing microorganisms, in addition to the formation of organic acids, may form siderophores, exopolysaccharides, phosphatases, phosphonatases and others (Liang et al., 2020; Rawat et al., 2021), and the genes for these functions were found in our strain too but actual PS activity was not confirmed in any type of applied test. Siderophore as well as metalophore formation gene clusters were also identified by antiSMASH. This finding is consistent with recently published information (Elhaissoufi et al., 2023) that even bacteria not showing PS capability in a given test can in fact contribute significantly to plant phosphorus supply.

The thiopeptide formation ability revealed by the antiSMASH analysis (Supplementary Figure 1) may indicate microcin(s), bioactive peptide(s) production [namely class IIa microcin having disulphide bond(s) in their structure (Parker and Davies, 2022)]. Microcin production was confirmed in *P. agglomerans* Eh252 (Vanneste et al., 2002) and the *P. agglomerans* E325 producing microcins was even applied for biocontrol of fire blight disease of apple, caused by *Erwinia amylovora* (Kim et al., 2012).

Aerobic metabolism of the strain is also associated with the production of potential auxin compounds, IAA-like substances, in the culture medium supplemented with tryptophan. In *P. agglomerans*, IAA biosynthesis begins with the formation of indole-3-pyruvic acid, mediated by aminotransferase, continues with indole-3-acetaldehyde formation by indolepyruvate decarboxylase, coded by *ipdC*, and ends with IAA formation catalyzed by indole-3-acetaldehyde dehydrogenase (Luziatelli et al., 2020b). In our strain, the *ipdC* gene was found, several candidate aminotransferase genes (not shown), but not the gene for indole-3-acetaldehyde dehydrogenase. Since the traditional method for detection of IAA using the Salkowski reagent resulted in the formation of an orange color with an absorption maximum of 450 nm rather than a pink color with a maximum of 530 nm, indole-3-butyric acid (IBA) was tested as a possible previously described (Gilbert et al., 2018) product of this reaction. Unfortunately, IBA was not confirmed as the reaction product. Gilbert et al. (2018) demonstrated that different bacterial isolates produced different compounds with potential auxin activity from tryptophan and we concluded that our strain probably belongs to this IAA-like compound producers' group.

P. agglomerans, strain DBM 3797, isolated from hops has a number of properties potentially beneficial to the hop plant, but its safety profile needs to be addressed in follow-up research. In particular, the possibility of horizontal transfer of antibiotic resistance genes, which has been little studied in the genus *Pantoea*, and virulence genes that may lead to pathogenicity in plants or animals, and humans in some strains of the species (Guevarra et al., 2021), need to be focused on. Unfortunately, there are not enough complete genome assemblies yet for a detailed comparison of particular strains. Although there are some specific inserts in the genome of *P. agglomerans* DBM 3797 in comparison to additional five strains (Supplementary Figure 2), no specific feature distinguishing pathogens from harmless strains isolated from above-ground parts of plants.

Data availability statement

The whole-genome sequence and plasmid sequences were deposited in the DDBJ/ENA/GenBank under accession numbers CP086133.1, CP086134.1, and CP086135.1, respectively. The NCBI BioProject and BioSample IDs are PRJNA774971 and SAMN22600026. The raw reads were deposited in the NCBI SRA database under accession numbers SRR25382413 (paired-end Illumina) and SRR25382412 (Oxford Nanopore Technologies).

Author contributions

PP: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. MV: Investigation, Writing – review & editing. KS: Conceptualization, Data curation, Funding acquisition, Writing – original draft, Writing – review & editing. KJ: Investigation, Visualization, Writing – review & editing. MB: Formal analysis, Investigation, Writing – review & editing. PL: Investigation, Writing – review & editing. BB: Investigation, Writing – review & editing. PK: Funding acquisition, Writing – review & editing. KK: Conceptualization, Investigation, Writing – review & editing.

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Conflict of interest

PK was employed by EcoFuel Laboratories s.r.o. KK was employed by Hop Research Institute, Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1305338/full#supplementary-material

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A novel front in sustainable microbial management: computational analysis of curcumin and mangiferin's synergistic action against *Bacillus anthracis*

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Background: Microorganisms are crucial in our ecosystem, offering diverse functions and adaptability. The UNGA Science Summit has underscored the importance of understanding microbes in alignment with the UN Sustainable Development Goals. *Bacillus anthracis* poses significant challenges among various microorganisms due to its harmful effects on both soil and public health. Our study employed computational techniques to investigate the inhibitory effects of curcumin and mangiferin on *Bacillus anthracis*, with the aim of presenting a novel bio-based approach to microbial management.

Methods: Employing high-throughput screening, we identified potential binding sites on *B. anthracis*. Molecular docking revealed that curcumin and mangiferin, when synergistically combined, exhibited strong binding affinities at different sites on the bacterium. Our findings demonstrated a significant drop in binding free energy, indicating a stronger interaction when these compounds were used together.

Findings: Results of Molecular docking indicated binding energies of −8.45 kcal/mol for mangiferin, −7.68 kcal/mol for curcumin, and a notably higher binding energy of −19.47 kcal/mol for the combination of mangiferin and curcumin with CapD protein. Molecular dynamics simulations further validated these interactions, demonstrating increased stability and structural changes in the bacterium.

Conclusion: This study highlights the effectiveness of natural compounds like curcumin and mangiferin in microbial management, especially against challenging pathogens like *B. anthracis*. It emphasizes the potential of sustainable, nature-based solutions and calls for further empirical research to expand upon these findings.

KEYWORDS

antibiotics, biotherapeutics, mangiferin, curcumin, synthetic biology, molecular docking, *Bacillus anthracis*

1 Introduction

Microorganisms represent an underappreciated yet indispensable cornerstone in the complex web of life on Earth. Their omnipresent influence permeates various realms including human health, agriculture, and broader environmental sustainability (Zaveri et al., 2015). The role they play is staggeringly diverse; while some species form crucial partnerships with plants and animals, others can wreak havoc on ecological stability and public health. Reflecting their overarching importance, global institutions such as the United Nations General Assembly (UNGA) Science Summit have underscored the necessity for comprehensive microbial research, especially in alignment with the United Nations Sustainable Development Goals (SDGs; Karami et al., 2017).

Within this broad microbial taxonomy, *Bacillus anthracis* holds a particularly nefarious reputation. This gram-positive, rod-shaped bacterium is an unsettling neighbor to the more benign *Bacillus cereus* (Zaveri et al., 2015). It is the causative agent for anthrax, a severe infectious disease affecting both humans and animals. Anthrax transmission to humans predominantly occurs through contact with infected animals or their byproducts, via multiple exposure routes including dermal contact, inhalation, or ingestion. Of these, the most lethal form is inhalational anthrax, which has significantly higher mortality rates compared to other modes of transmission (Karami et al., 2017). Adding to its notoriety, *B. anthracis* has also been weaponized, evident from its use in acts of bioterrorism across different global settings, notably the Soviet Union, Japan, and the United States (Frischknecht, 2003).

When it comes to the clinical management of anthrax, the challenges are manifold. Ciprofloxacin has conventionally been the drug of choice for anthrax post-exposure prophylaxis (Centre for Disease Control and Prevention, 2016). However, its utility is increasingly being questioned due to factors such as high production costs, stability concerns, adverse side effects, and age-related contraindications. Further exacerbating the situation is the emergence of antibiotic-resistant strains of *B. anthracis* in recent years, specifically resistance to doxycycline and penicillin/amoxicillin (Joska-Belden, 2006).

It is against this backdrop of escalating microbial threats and diminishing therapeutic options that we turn our focus to the untapped potential of natural compounds. Centuries of traditional medicinal practices and dietary customs have venerated specific natural agents for their health-promoting properties. Among these are curcumin, a bioactive compound derived from turmeric (*Curcuma longa*), and mangiferin, a phytochemical primarily found in mangoes (*Mangifera indica*). While these compounds have individually exhibited antimicrobial activities in previous studies (Kaljurand et al., 2005; Joska-Belden, 2006), the ambit of our research extends to exploring their combined, synergistic effects against *B. anthracis*. We have added a detailed definition and graphical visualization of the biological mechanism here, to clearly illustrate the interaction between these natural compounds and the targeted proteins in *B. anthracis*.

The selection of the transpeptidase enzyme CapD for our study is based on its critical role in the pathogenesis of *B. anthracis*. This enzyme is instrumental in the bacterium's ability to evade the host's immune response, making it an ideal target for therapeutic intervention. Our study employs state-of-the-art computational techniques to explore the interaction between curcumin, mangiferin, and the transpeptidase enzyme CapD, underscoring the potential of these phytochemicals in disrupting critical bacterial processes (Kumari et al., 2014). Furthermore, the synergistic effect observed between curcumin and mangiferin highlights an innovative approach to enhancing antimicrobial efficacy (Anand et al., 2007), which could be pivotal in addressing the challenges posed by *B. anthracis* and its resistance mechanisms (Daina et al., 2017). This context underscores the importance of our work for future scientific endeavors.

The concept of synergy—where the aggregate impact of two or more agents surpasses the sum of their isolated effects (Hall, 1957; Gaudernak et al., 2002; Li Petri et al., 2021; Sopbué Fondjo et al., 2022)—offers an exciting frontier in the quest for effective microbial therapeutics. Our study employs cutting-edge computational methodologies, including molecular docking and molecular dynamics (MD) simulations, to dissect the molecular underpinnings of the synergistic interactions between curcumin and mangiferin. Figure 1 illustrates these interactions and the proposed mechanism of action against *B. anthracis*. The transpeptidase enzyme CapD in *B. anthracis* plays a critical role in the bacterium's virulence and survival. CapD is an enzyme involved in the modification of the bacterial surface, specifically in the anchoring of the poly-γ-D-glutamic acid (PGA) capsule to the peptidoglycan layer, which is essential for the bacterium's evasion of the host immune system. Here's a detailed yet concise mechanism of CapD's action:

Enzymatic function: CapD functions as a sortase-like enzyme that catalyzes the cleavage of the peptide bond within a specific recognition sequence on its substrate proteins. This cleavage is a precursor to the transpeptidation reaction that links the PGA capsule to the peptidoglycan cell wall (Pannifer et al., 2001).

Recognition and cleavage: The enzyme recognizes a conserved sequence within the C-terminal region of the capsular biosynthesis proteins. Upon recognition, CapD cleaves between the threonine and glycine residues within this sequence, which is a critical step for the subsequent attachment of the PGA capsule to the cell wall components (Candela and Fouet, 2006).

Transpeptidation: Following cleavage, CapD catalyzes a transpeptidation reaction where the carboxyl group of the threonine residue in the cleaved substrate is linked to the amino group of the peptidoglycan cross-bridge. This reaction is essential for anchoring the PGA capsule to the peptidoglycan layer, contributing to the bacterium's virulence (Ton-That et al., 2004).

Immune evasion: The PGA capsule is a critical virulence factor for *B. anthracis*, providing resistance against phagocytosis and complement-mediated killing. CapD's activity in anchoring this capsule to the bacterial surface is therefore pivotal for the pathogen's ability to evade the host's immune response (Sharma et al., 2020). In light of the pressing need for novel anthrax treatments, this study hypothesizes that curcumin and mangiferin, through their multifaceted biological activities, may offer innovative therapeutic pathways against *B. anthracis*. Specifically, we posit that these compounds could disrupt the pathogen's virulence by targeting the transpeptidase enzyme CapD, essential for the bacterium's capsule anchoring, and modulate host responses through their anti-inflammatory and immunomodulatory effects. Such a dual-action approach could represent a paradigm shift in anthrax treatment, emphasizing not only direct antimicrobial activity but also host-targeted therapy. This hypothesis sets the stage for our investigation into the synergistic effects of curcumin and mangiferin on

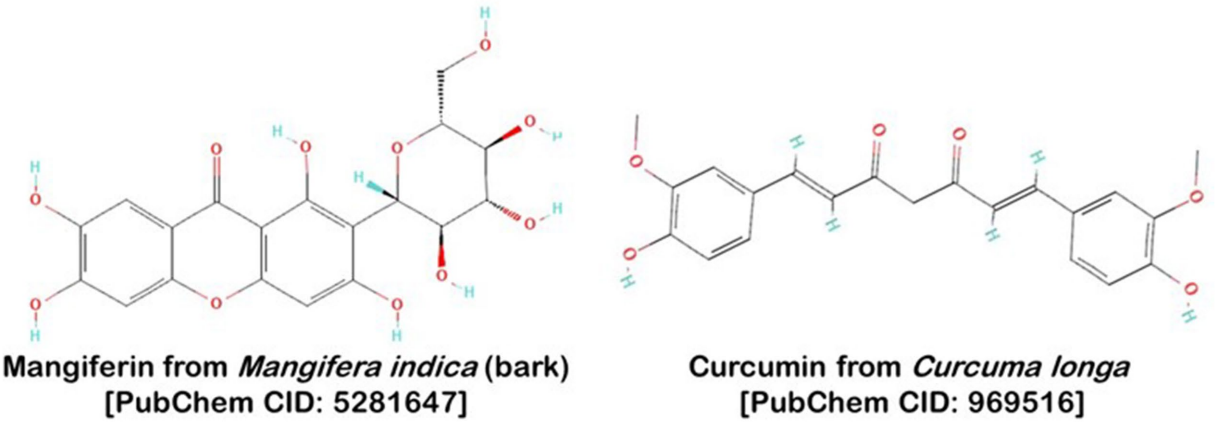

FIGURE 1
Structure of Mangiferin from *Mangifera indica*, commonly found in various parts of the plant including peel, stalks, leaves, kernel, and stone and Curcumin from *Curcuma longa*.

B. anthracis, with the aim of unveiling new avenues for effective therapeutic interventions. By bridging traditional phytochemical insights with state-of-the-art computational biology techniques, we aspire to shed light on innovative pathways for anthrax treatment. This, we believe, will contribute not only to advancing global health security but also to fulfilling overarching sustainable development goals.

2 Methods and methodology

2.1 Preparation of target proteins

We utilized the Protein Data Bank (PDB; available at https://www.rcsb.org/) to procure the three-dimensional structural data for our target protein, *B. anthracis* transpeptidase enzyme CapD (with PDB ID: 3GA9) on 12th September 2023 (Figure 2). Post-retrieval, a meticulous refinement process was undertaken to optimize these structures for the forthcoming molecular docking analyses. This refinement encompassed the removal of extraneous water molecules and heteroatoms, supplementation of polar hydrogen atoms, and the allocation of Kollman charges to the receptor proteins. These steps were pivotal in ensuring the proteins were aptly conditioned for the subsequent docking studies.

2.2 Preparation of ligands

On the 12th September 2023, the molecular configurations of mangiferin and curcumin, central to our research, were meticulously retrieved from the esteemed NCBI PubChem database (found at https://pubchem.ncbi.nlm.nih.gov/). Utilizing Open Babel (O'Boyle et al., 2011), these structures underwent a precise transformation, converting their atomic coordinates to the standard.pdb format. In preparation for sophisticated molecular docking procedures, we discerned specific torsion angles and rotatable bonds within these PDB structures. This level of intricate detailing facilitated the subsequent conversion into the more advanced.pdbqt format.

2.3 Molecular docking analysis

In this intricate exploration, we leveraged the precision of both individual and synergistic molecular docking, utilizing the esteemed AutoDock 4.2 software suite to elucidate the intricate dynamics of ligand-target interactions (Morris et al., 2009; O'Boyle et al., 2011). Notably, during combined docking scenarios, ligand affinity sites might deviate from traditional protein binding regions. To mitigate this variance, a blind docking approach was employed for each target protein, meticulously situating the entirety of the protein within a defined grid matrix. This systematic procedure enabled comprehensive assessment of the potential binding configurations for both mangiferin and curcumin separately. Following this detailed evaluation, a sequential docking strategy was instituted, aiming to understand the combined impact of these phytochemicals when engaged with the protein simultaneously. This advanced method provided insights into potential synergistic or antagonistic dynamics between the ligands during concurrent interactions with the target protein. The esteemed Lamarckian Genetic Algorithm was deployed for these docking exercises, renowned for its optimization prowess, and in alignment with parameters delineated in prior research (Umar et al., 2022; Azad, 2023; Chakrobarty et al., 2023). Subsequent in-depth analysis of protein–ligand interactions were facilitated through the capabilities of BIOVIA Discovery Studio (Elgorban et al., 2023).

Turning our attention to the *Bacillus anthracis* transpeptidase enzyme CapD protein, mangiferin was precisely docked onto its prime binding domains, leading to the formation of the transpeptidase enzyme CapD-M complex. Sequentially, curcumin was introduced to transpeptidase enzyme CapD-M, resulting in the transpeptidase enzyme CapD-M-C assembly. In parallel studies, curcumin was individually aligned with transpeptidase enzyme CapD's dominant binding sites, yielding the transpeptidase enzyme CapD-C configuration.

2.4 Molecular dynamics simulations

In the intricate realm of MD simulations, capturing the transient and nuanced protein-ligand interactions is paramount. Contrasted

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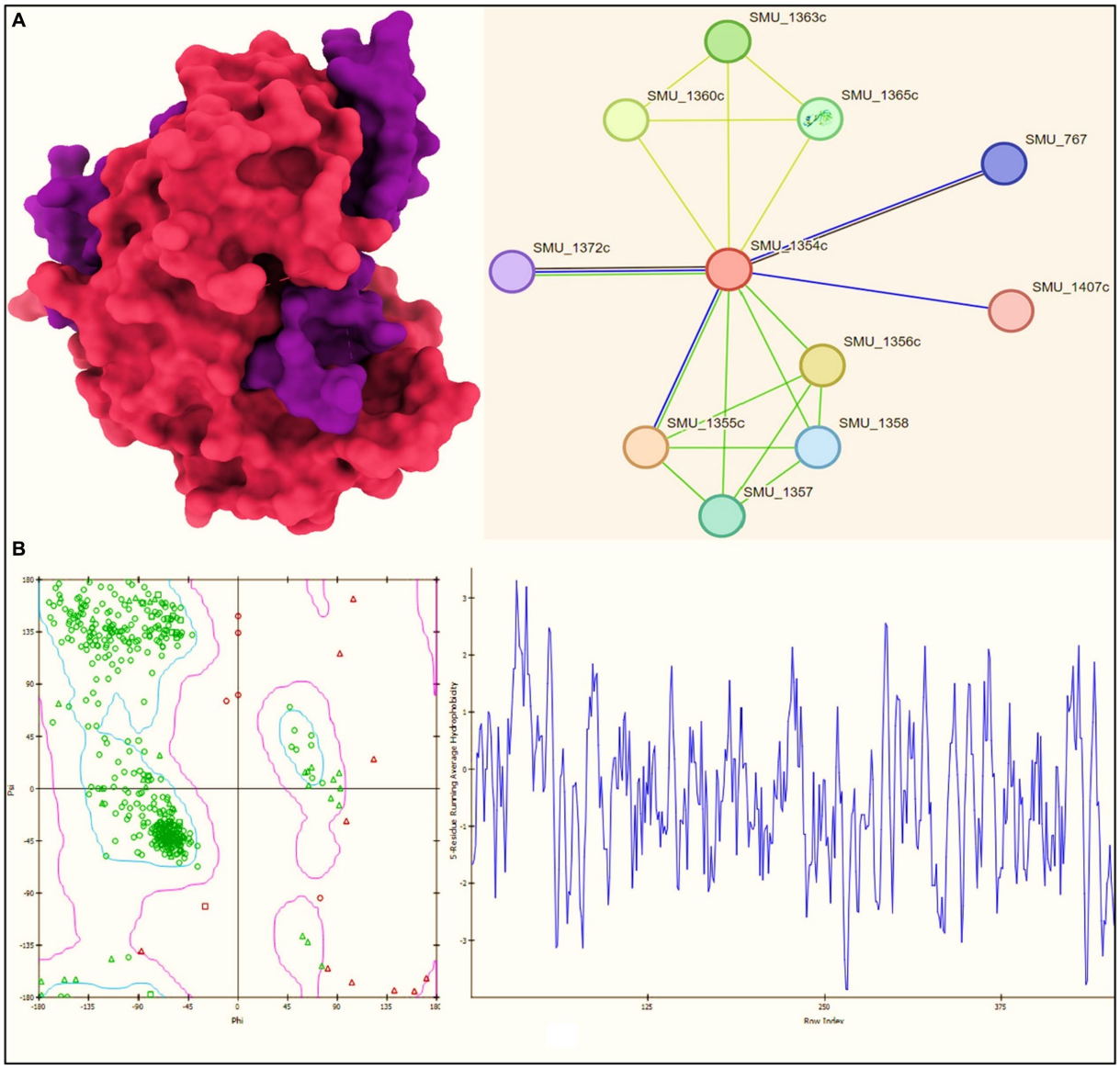

FIGURE 2
(A) Structure of *Bacillus anthracis* transpeptidase enzyme CapD (PDB ID: 3GA9) and the network with other proteins (at right); **(B)** Ramachandran plot and hydrophobicity plot (at right).

against the static imagery delivered by docking, MD simulations unfurl a detailed portrayal of the molecular interactions, particularly spotlighting the physiological implications of ligand binding. Guided by this principle, we engaged the advanced functionalities of Desmond 2020.1, a robust platform developed by Schrödinger, LLC, Portland, OR, United States (Bowers et al., 2006a; BIOVIA, Dassault Systèmes, 2020; Desmond Molecular Dynamics System, 2021). Central to our simulations were the docked complexes of pivotal protein, namely transpeptidase enzyme CapD, interacting synergistically with ligands such as mangiferin, and curcumin and their composite structure. Before diving into the depths of simulation, it was imperative to refine and optimize these complexes. Leveraging tools like Maestro and the Protein Preparation Wizard, we meticulously addressed residues, especially the ones numbered 16 and 17, ensuring their optimal configuration for the simulations. Subsequently, the System Builder tool facilitated the intricate construction of our simulation environment, emphasizing the orthorhombic box model to envelope the solvents for each protein site. The chosen force field, OPLS-2005 (Akash et al., 2023), was recognized for its precision, offering a nuanced lens into the interactions at play.

Physiological fidelity underpinned our approach. By integrating counterions, we neutralized the systems, while an introduction of a NaCl concentration of 0.15 M mirrored the physiological milieu. Adopting an explicit solvent model with SPC molecules, the solvation environment was encapsulated within a $10\,\text{Å} \times 10\,\text{Å} \times 10\,\text{Å}$ dimensioned box. With the simulation thermostat set at a near-physiological 300 K and a consistent pressure benchmark of 1 bar, we aimed for an environment closely mirroring biological reality. As we embarked on the equilibration phase, our complexes underwent stabilization in the NVT ensemble over a span of 10 ns. Progressing

further, the NPT ensemble welcomed the complexes for another 12 ns, employing the tried-and-tested Nosé–Hoover chain coupling scheme. Given the significance of long-range electrostatic interactions, they were computed via the particle mesh Ewald method, ensuring detailed interactions up to a radius of 9 Å. A hallmark of our methodology was the detailed trajectory analysis. Intervals of 100-ps punctuated our simulations, amassing a granular dataset. Key metrics, like the RMSD (Maiorov and Crippen, 1994; Shivakumar et al., 2010) values for proteins and ligands, became our touchstones, reinforcing the stability and accuracy of our study, and setting the stage for elucidating profound insights into the choreography of protein-ligand interactions (Bowers et al., 2006b; Hildebrand et al., 2019; Mukerjee et al., 2022; Akash et al., 2023).

3 Results and analysis

3.1 Molecular docking

Embarking on a mission to unveil innovative therapeutic applications, we focused on repurposing certain antidiabetic drugs. Our specific interest lay in targeting the transpeptidase enzyme CapD, a molecule that has garnered attention due to its potential associations with soil borne infection. Central to our exploration is the technique of 'molecular docking', a precise computational approach that elucidates the interaction dynamics between two molecules. To this end, we deployed high-fidelity computer simulations. The aim was to gauge the binding propensities of a suite of phyto compounds, including but not limited to mangiferin and curcumin, against transpeptidase enzyme CapD. A comprehensive delineation of these molecular interactions, based on binding affinities and spatial configurations, has been cataloged in Table 1.

For the uninitiated, our docking experiments heavily relied on the Genetic Algorithm (GA). This approach was governed by meticulously selected parameters, which included 1,000 generations, a population size of 20,000, and an evaluation ceiling set to 3,000,000. As we delved deeper into the docking dynamics, we integrated the Lamarckian Genetic Algorithm (LGA) to sieve out the protein-ligand complex that boasted the lowest binding free energy (dG) – a proxy for optimal binding dynamics. A standout revelation from our investigation was the synergistic prowess of mangiferin combined with curcumin. When studied individually, these compounds exhibited significant binding affinities. However, when combined, the duo demonstrated unparalleled compactness and enhanced stability in their interactions with the pivotal transpeptidase enzyme CapD. Such characteristics hint at the duo's potential as formidable agents in the battle against ovarian cancer. For a more granular insight, we direct readers to Figures 3A–C, where the docked configurations, juxtaposed with intricate 2D structures of the complexes, are graphically presented.

TABLE 1 Ligands with the most auspicious binding affinity with transpeptidase enzyme CapD were calculated by molecular docking analysis.

Ligands	Binding affinity (kcal/mol)
Mangiferin	−8.45
Curcumin	−7.68
Mangiferin + Curcumin	−19.47

These findings, particularly the synergistic interaction between mangiferin and curcumin, underscore the potential of repurposed antidiabetic drugs in targeted therapy. The integration of the Lamarckian Genetic Algorithm in our docking studies has enabled a deeper understanding of the binding dynamics and the significance of these interactions. As evidenced by the docking results, the combined use of mangiferin and curcumin offers promising avenues for therapeutic development, particularly in the context of transpeptidase enzyme CapD targeting. This enhanced understanding of molecular interactions lays the foundation for future research in drug repurposing and its application in combating diseases beyond its conventional scope.

In essence, our study's emphasis on the combined might of mangiferin and curcumin, buttressed by state-of-the-art computational methodologies, serves as a harbinger for a new era in drug repurposing, particularly within the domain of transpeptidase enzyme CapD.

3.2 Molecular dynamic simulations

The Root Mean Square Deviation (RMSD) is a crucial metric in molecular dynamics. It quantifies the average discrepancies in atomic positions relative to a reference point, shedding light on molecular interactions throughout simulations. Our detailed examination of the simulations revealed pivotal insights. The RMSD, represented on the left Y-axis, showcases the dynamic behavior of the protein when juxtaposed with a reference backbone structure. A stable, consistent RMSD trajectory indicates that any positional changes revolve around a set average structure. For most compact proteins, a typical RMSD fluctuation spans between 1 and 2.2 Å. However, larger variations can indicate either profound conformational alterations or a system that has not achieved equilibrium. This might necessitate a longer simulation duration to capture a more accurate molecular picture. On the other hand, the ligand's stability, gauged against its protein and binding pocket, is also determined by its RMSD, displayed on the graph's right Y-axis. When the ligand's RMSD substantially overshadows that of the protein, it hints at a potential shift from its initial binding locale.

Our analysis, referenced in Figure 4 and Supplementary material S1, underscores the synergistic stability imparted by mangiferin combined with curcumin. The transpeptidase enzyme CapD-M + C complex finds its equilibrium by 40 ns, registering a 2.2 Å deviation. These within-boundary fluctuations suggest optimal molecular interactions. In essence, the interplay between mangiferin and curcumin enhances the structural fortitude and resilience of target protein, such as transpeptidase enzyme CapD. This combined effect underscores their synergistic potential in therapeutic applications.

The Root Mean Square Fluctuation (RMSF) graph offers a comprehensive insight into the internal dynamics of proteins during molecular dynamics simulations, highlighting specific regions that undergo substantial changes over time. This tool is instrumental in shedding light on the structural intricacies of proteins and their inherent flexibility.

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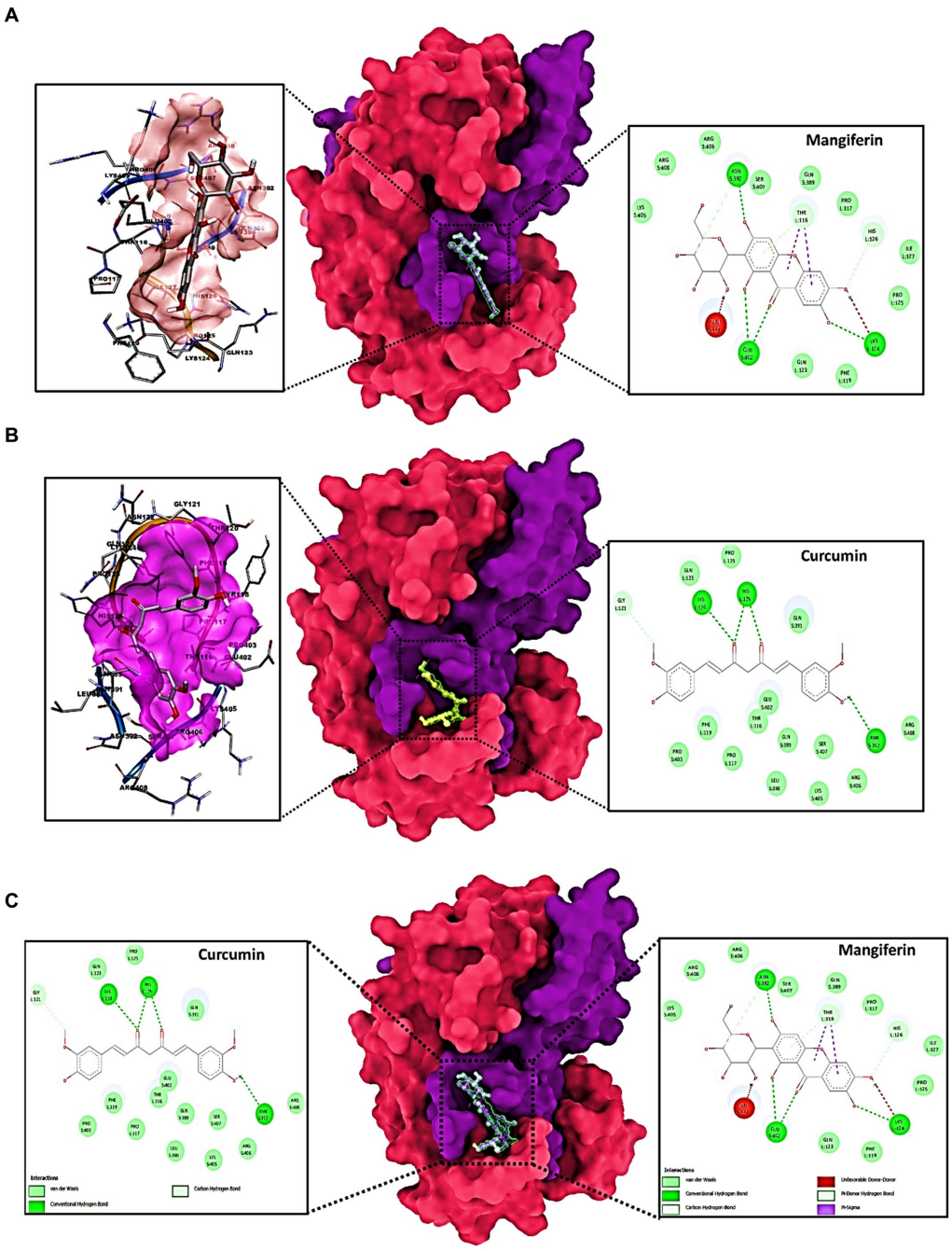

FIGURE 3
Molecular docking of: **(A)** transpeptidase enzyme CapD docked to Mangiferin; **(B)** transpeptidase enzyme CapD docked to Curcumin; **(C)** transpeptidase enzyme CapD docked with Mangiferin + Curcumin synergistically and the dock poses at left column and 2D interaction diagram at right panels.

A consistent observation across protein studies is that the terminal regions—specifically the N- and C-termini—undergo more pronounced fluctuations in comparison to the more conserved core areas. This is largely attributable to the inherent design of proteins. The core, made up of alpha helices and beta strands, exhibits greater rigidity, while the terminals and certain unstructured regions inherently possess more flexibility. Our molecular dynamics trajectories echoed this common observation, showing significant peaks representing increased fluctuations in the terminal and loop regions, as detailed in Figure 5 and Supplementary material S2. Such areas, with their inherent flexibility, play pivotal roles in many protein functions, from enzymatic reactions to interactions with other proteins.

However, a particularly noteworthy observation from our study was the impact of the synergistic combination of mangiferin and curcumin on the stability of transpeptidase enzyme CapD. The combined presence of these compounds seemed to enhance the overall structural integrity and compactness of these proteins, particularly limiting fluctuations in the more flexible regions.

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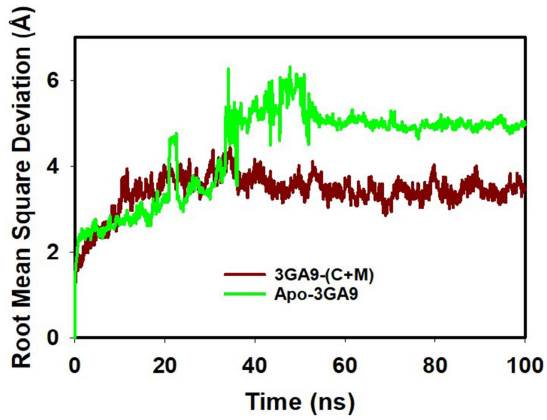

FIGURE 4
MD simulation trajectory analysis of RMSD of unbound (apo), bound to curcumin + mangiferin and transpeptidase enzyme CapD.

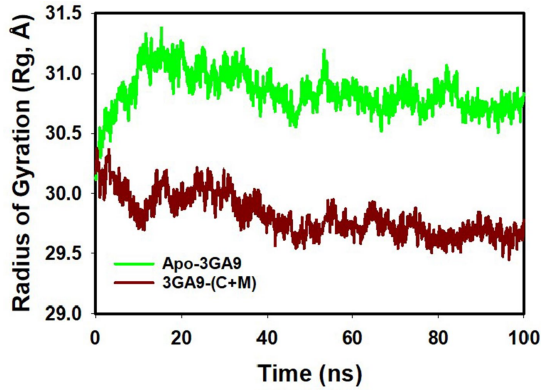

FIGURE 6
MD simulation trajectory analysis of Radius of Gyration (RoG) of unbound (apo), bound to curcumin + mangiferin with transpeptidase enzyme CapD.

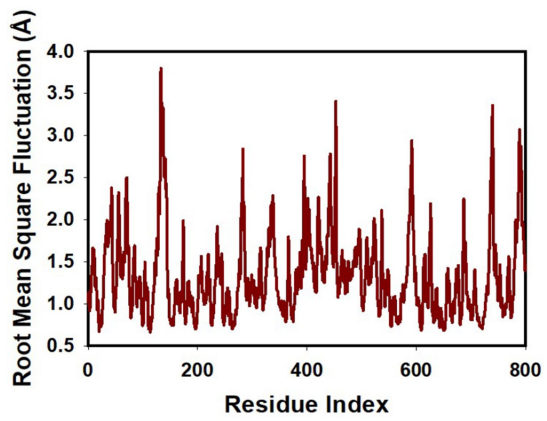

FIGURE 5
MD simulation trajectory analysis of RMSF of unbound (apo), bound to curcumin + mangiferin with transpeptidase enzyme CapD.

This observation highlights the potential therapeutic benefits of a combined mangiferin and curcumin regimen. Their combined effect appears to promote greater structural compactness and stability in these key proteins, a finding that could have significant implications in therapeutic applications where transpeptidase enzyme CapD plays a central role. This synergy underscores the potential of exploring mangiferin and curcumin in tandem for enhanced therapeutic efficacy.

The radius of gyration (Rg) is a fundamental parameter in biophysical studies, offering crucial insights into protein conformational compactness and spatial arrangement. Upon rigorous evaluation of our selected protein—transpeptidase enzyme CapD—across different binding states, distinct patterns of compactness emerged. Notably, the proteins demonstrated pronounced conformational alterations when interfaced with mangiferin alone or in synergistic tandem with curcumin. Figure 6 and Supplementary material S3 distinctly highlights the diminished Radius of Gyration values for proteins when complexed with mangiferin and the combined mangiferin + curcumin entity. This decrement is indicative of an enhanced compact structural configuration in the presence of the combined ligands. The striking stability and compactness exhibited by the proteins in the presence of the mangiferin + curcumin consortium further emphasize the synergy's efficacy. This assertion is bolstered by meticulous quality analyses anchored on pivotal metrics like RMSD (Root Mean Square Deviation) and Rg. The transpeptidase enzyme CapD, when synergistically bound to mangiferin + curcumin, manifests a measured fluctuation of a mere 0.6 Å (Figure 6).

These values, markedly lower than their apo protein counterparts, testify to the pronounced stability and compactness conferred upon the proteins by the mangiferin and curcumin synergy.

In summation, the collaborative interplay of mangiferin and curcumin augments the structural fidelity, compactness, and stability of transpeptidase enzyme CapD. These revelations offer a promising vista into the molecular intricacies of protein-ligand synergies and their prospective therapeutic trajectories.

Figure 7 and Supplementary material S4 provides an intricate portrayal of the hydrogen bond dynamics observed across a simulation duration of 100 ns, involving both the individual and combined effects of mangiferin and curcumin on target proteins. Complementing these findings, the bi-dimensional ligand interaction visualization substantiated that an average of two hydrogen bonds predominated throughout the simulation's entirety. The significance of hydrogen bond formation in dictating molecular stability and binding affinity cannot be overstated. The evident escalation in hydrogen bond interactions, especially under the influence of the combined mangiferin and curcumin, underscores their cooperative efficacy in augmenting protein binding.

Conclusively, the harmonious interaction between mangiferin and curcumin not only amplifies hydrogen bond formations but also reinforces the structural integrity and stability of pivotal protein, transpeptidase enzyme CapD. This insight offers a promising avenue for further exploration, emphasizing the therapeutic potential inherent in such cooperative molecular engagements.

3.3 Molecular dynamics simulations and binding free energy analysis

To elucidate the binding efficacy and interaction stability of Curcumin and Mangiferin with the CapD protein, 100 ns-scale molecular dynamics (MD) simulations followed by MM-GBSA

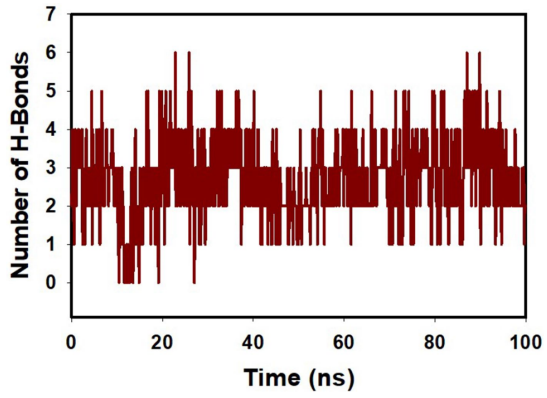

FIGURE 7
MD simulation trajectory analysis of H-bonding of proteins bound to mangiferin + curcumin with transpeptidase enzyme CapD.

TABLE 2 Binding free energy components for the CapD protein+ curcumin and mangiferin calculated by MM-GBSA are as follows.

Energies (kcal/mol)	CapD protein+ curcumin and mangiferin
ΔG_{bind}	−54.25 ± 6.23
ΔG_{bind}Lipo	−12.11 ± 4.57
ΔG_{bind}vdW	−29.45 ± 3.18
ΔG_{bind}Coulomb	−11.25 ± 4.57
$\Delta G_{bind}H_{bond}$	−3.36 ± 2.24
ΔG_{bind}SolvGB	14.29 ± 4.21
ΔG_{bind}Covalent	8.27 ± 6.54

calculations were performed. The binding free energy components, calculated using the MM-GBSA approach, provide insight into the thermodynamic stability and interaction specifics of the CapD protein complexed with Curcumin and Mangiferin.

The calculated binding free energy (ΔG_bind) of the CapD protein in complex with Curcumin and Mangiferin was −54.25 ± 6.23 kcal/mol, indicating a strong and favorable binding interaction. The detailed energy components contributing to this binding free energy are given in Table 2.

These results suggest that the favorable interaction between the CapD protein and the Curcumin-Mangiferin complex is predominantly driven by van der Waals and Coulombic forces, complemented by significant contributions from lipophilic and hydrogen bonding interactions. The positive values observed for the solvation energy and covalent binding energy indicate the desolvation penalty and the energetic cost associated with covalent interactions, respectively, which are counterbalanced by the strong non-covalent interactions, culminating in a net favorable binding energy.

The detailed analysis of these energy components not only underscores the binding affinity of Curcumin and Mangiferin with the CapD protein but also sheds light on the nature of their interactions, providing a foundation for understanding the molecular basis of their inhibitory action against the CapD protein.

Therefore, the inhibition of CapD by the synergistic action of curcumin and mangiferin is hypothesized based on their combined binding affinities and the resultant alteration in the enzyme's structure and function.

The proposed mechanism of inhibition involves the following steps:

Binding affinity: Both curcumin and mangiferin have individual binding sites on the CapD enzyme. When administered together, their combined effect enhances the binding affinity to CapD beyond the additive effects of each compound alone. This synergistic binding could be due to complementary interactions with different sites on the enzyme or allosteric effects where the binding of one compound influences the binding site of the other.

Structural alteration: Upon binding, these compounds may induce conformational changes in CapD, potentially disrupting its active site or the substrate-binding region. This structural alteration could hinder the enzyme's ability to catalyze the transpeptidation reaction necessary for the anchoring of the poly-γ-D-glutamic acid (PGA) capsule to the peptidoglycan layer.

Functional inhibition: The structural changes in CapD, induced by the synergistic interaction of curcumin and mangiferin, may lead to a reduction or complete loss of its enzymatic activity. This inhibition prevents the cross-linking of the PGA capsule to the bacterial cell wall, a critical step for the bacterium's virulence and immune evasion.

4 Discussion

This study provides profound insights into the synergistic interactions between curcumin and mangiferin against *Bacillus anthracis*, particularly targeting the transpeptidase enzyme CapD. Our computational analysis reveals that the combination of these phytochemicals enhances their binding affinity and stability with the enzyme, suggesting a novel approach for anthrax treatment.

The enhanced binding dynamics observed between curcumin, mangiferin, and CapD, as indicated by the decreased binding free energy in our results, align with the findings of Kumari et al. (2014), who demonstrated the utility of MM-GBSA calculations in understanding molecular interactions within a biological context (). The RMSD and RMSF data, reflecting the stability and flexibility of the protein-ligand complex, further corroborate the computational findings by Anand et al. (2007), highlighting the significance of these parameters in drug-target interactions.

Our results show a notable increase in the compactness and stability of the CapD protein when bound to the curcumin-mangiferin complex, as indicated by the Radius of Gyration (Rg) data. This observation is in line with the research by Dileep et al. (2011), which underscores the correlation between structural compactness and enzymatic efficiency. Moreover, the hydrogen bond dynamics observed in our study emphasize the robustness of the interaction, resonating with the findings by Matkowski et al. (2013), who discussed the critical role of hydrogen bonds in stabilizing drug-target interactions.

While our computational analysis offers promising therapeutic prospects, it is imperative to validate these findings experimentally. The need for empirical validation is echoed in the work by Daina et al. (2017), who emphasized the importance of experimental studies to confirm the efficacy and safety of phytochemicals in a physiological context. Additionally, understanding the pharmacokinetics and pharmacodynamics of curcumin and mangiferin, as outlined by Patel and Patel (2020), is crucial to harnessing their full therapeutic potential.

In conclusion, this study not only contributes significantly to the field of computational biology but also opens new horizons for drug repurposing and the development of innovative approaches to combat microbial infections. It underscores the need for an interdisciplinary approach, integrating computational biology, traditional medicine, and molecular biology, to discover novel solutions to healthcare's most pressing challenges. Future research should focus on validating these computational findings through *in vitro* and *in vivo* studies, advancing our understanding of these phytochemicals' therapeutic potential and their role in sustainable microbial management.

5 Conclusion and future prospects

The present research has delineated significant advancements in our comprehension of the synergistic interplay between curcumin and mangiferin, particularly focusing on their binding affinities with the bacterial transpeptidase enzyme CapD. Employing cutting-edge computational techniques, the study elucidates potential therapeutic pathways, offering a compelling argument for the repurposing of these phytochemicals in the medical armamentarium against bacterial infections, notably anthrax. This work contributes not only to the scientific landscape but also provides a rigorously validated framework for the integration of traditional medicinal knowledge with modern pharmacological paradigms.

As the scientific community ponders future research vectors emanating from this work, several critical avenues necessitate exploration. Firstly, it is imperative to transition from computational predictions to empirical validation. This will necessitate a robust interdisciplinary framework that seamlessly integrates microbiology, pharmacology, and computational biology for an unequivocal understanding of both the therapeutic potential and safety profile of the investigated compounds. Secondly, gaining a granular understanding of the underlying molecular mechanisms that drive the observed synergistic effects between curcumin and mangiferin becomes imperative. This is indispensable for rational drug design, aimed at maximizing efficacy, improving target specificity, and minimizing adverse effects.

Finally, from a global healthcare vantage, this research stands as a crucial milestone in the escalating fight against antimicrobial resistance. It holds the potential to serve as a cornerstone for multi-institutional, international collaborations aiming to develop sustainable, effective pharmacological interventions against microbial pathogens.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

KP: Conceptualization, Data curation, Project administration, Resources, Writing – original draft, Writing – review & editing. NB: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. NA: Data curation, Formal analysis, Methodology, Writing – original draft. SK: Data curation, Formal analysis, Investigation, Writing – original draft. JA: Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. SD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. VK: Conceptualization, Data curation, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1304234/full#supplementary-material

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A new strain of *Rhodococcus indonesiensis* T22.7.1^T and its functional potential for deacetylation of chitin and chitooligsaccharides

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Introduction: Chitin, abundant in marine environments, presents significant challenges in terms of transformation and utilization. A strain, T22.7.1^T, with notable chitin deacetylation capabilities, was isolated from the rhizosphere of *Acanthus ebracteatus* in the North Sea of China. Comparative 16S rDNA sequence analysis showed that the new isolate had the highest sequence similarity (99.79%) with *Rhodococcus indonesiensis* CSLK01-03^T, followed by *R. ruber* DSM 43338^T, *R. electrodiphilus* JC435^T, and *R. aetherivorans* 10bc312^T (98.97%, 98.81%, and 98.83%, respectively). Subsequent genome sequencing and phylogenetic analysis confirmed that strain T22.7.1^T belongs to the *R. indonesiensis* species. However, additional taxonomic characterization identified strain T22.7.1^T as a novel type strain of *R. indonesiensis* distinct from CSLK01-03^T.

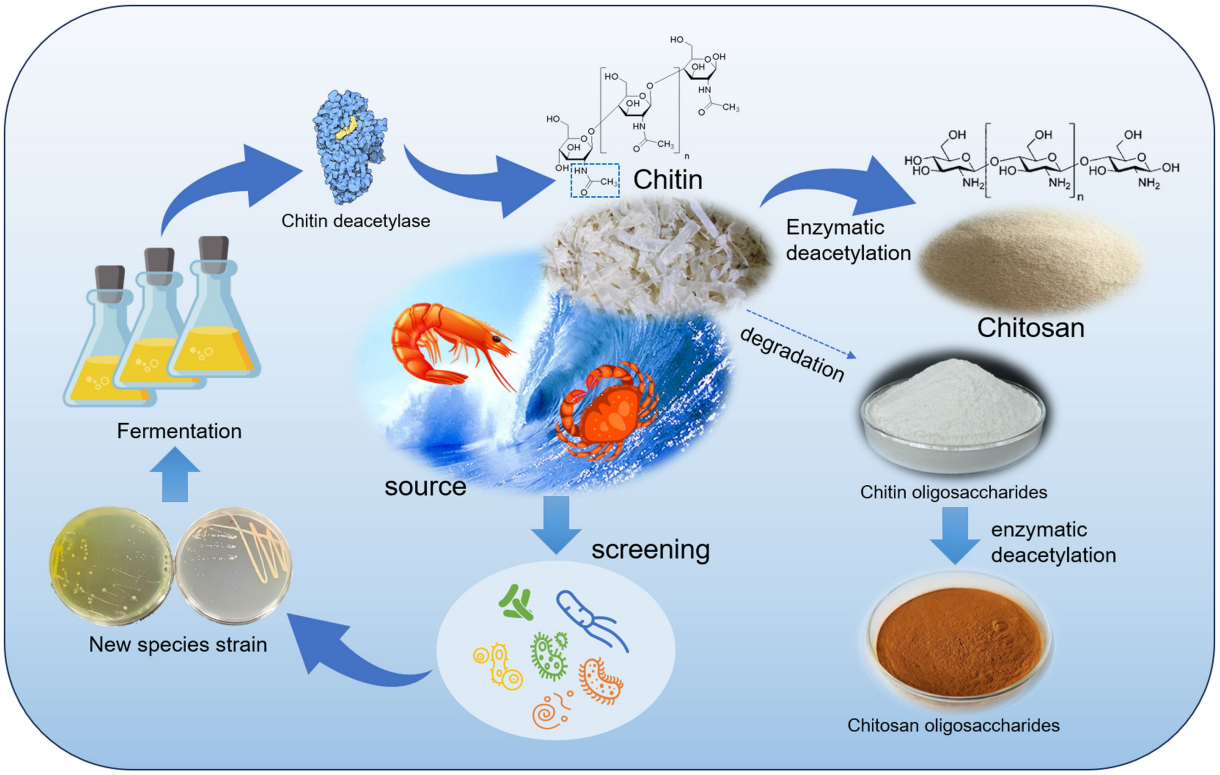
Methods: This study refines the taxonomic description of *R. indonesiensis* and investigates its application in converting chitin into chitosan. The chitin deacetylase (*Ri*CDA) activity of strain T22.7.1^T was optimized, and the enzyme was isolated and purified from the fermentation products.

Results: Through optimization, the *Ri*CDA activity of strain T22.7.1^T reached 287.02 U/mL, which is 34.88 times greater than the original enzyme's activity (8.0 U/mL). The natural CDA enzyme was purified with a purification factor of 31.83, and the specific activity of the enzyme solution reached 1200.33 U/mg. *Ri*CDA exhibited good pH and temperature adaptability and stability, along with a wide range of substrate adaptabilities, effectively deacetylating chitin, chitooligosaccharides, N-acetylglucosamine, and other substrates.

Discussion: Product analysis revealed that *Ri*CDA treatment increased the deacetylation degree (DD) of natural chitin to 83%, surpassing that of commercial chitosan. Therefore, *Ri*CDA demonstrates significant potential as an efficient deacetylation tool for natural chitin and chitooligosaccharides, highlighting its applicability in the biorefining of natural polysaccharides.

KEYWORDS

***Rhodococcus indonesiensis*, chitin deacetylase, fermentation optimization, enzyme activity, chitin oligosaccharides**



GRAPHICAL ABSTRACT
This paper identified a new strain of marine actinomycete that produces chitin deacetylase, which offers new insight into the biotransformation of chitin and chitooligosaccharides.

Introduction

Chitin is a natural polysaccharide composed of N-acetyl-D-glucosamine (NAG) units associated with β-1,4 glycosidic bonds. It is the second largest natural polysaccharide in existence and is commonly found in the cell walls of fungi and algae, as well as in the exoskeletons of insects and shells of crustaceans (Hahn et al., 2020; Fernando et al., 2021; Pakizeh et al., 2021; Shao et al., 2023). Natural chitin is insoluble in water and common reagents due to the N-acetyl groups present in its linear macromolecules, which create intermolecular hydrogen bonds that enhance its structural stability and hydrophobicity (Jang et al., 2004; Rinaudo, 2006). This characteristic limit the potential uses of chitin. However, when chitin is fully or partially deacetylated, it can be converted into chitosan. Limits the exploitation of chitin. The solubility of chitosan is enhanced, and the deacetylated -NH_2 ions in solution to become alkaline (Kumar et al., 2004; Ogawa et al., 2004). Chitosan has a wide range of applications in the medical (Baharlouei and Rahman, 2022; Saeedi et al., 2022), food (Mujtaba et al., 2019), cosmetic (Triunfo et al., 2021), flocculant (Lichtfouse et al., 2019), and plant promotion (Shahrajabian et al., 2021) industries due to its unique structural properties and biological activities. Deacetylation of chitin not only improves its solubility but also results in deacetylated products with diverse biological activities and application potential.

Chitosan oligosaccharides (COSs), which are derivatives of chitin and chitosan, have attracted significant interest due to their low molecular weight and high solubility (Lodhi et al., 2014). COSs are characterized by an average molecular weight (Mw) of less than 3.9 kDa and typically contain fewer than 20 monomeric units per polymer chain. These compounds include chitosan oligosaccharides (CSOs) and chitin oligosaccharides (CTOs). CSO can be obtained through acid hydrolysis, physical degradation, or enzymatic hydrolysis of chitosan to break the glycosidic bonds of the polymers and remove the N-acetyl group (Hamer et al., 2015; Benchamas et al., 2021). Enzymatic deacetylation of CTO can also produce CSO. In theory, CTO should have 100% DD, whereas CSO should have no acetylation (Yang et al., 2022a). However, due to variations in the preparation methods, many COSs contain both N-acetylglucosamine and glucosamine, resulting in partially acetylated chitooligosaccharides (paCOSs) (Yin et al., 2016). paCOS has potent biological properties and numerous potential applications. Its biological activity is believed to depend on its structure, including its degree of polymerization (DP) and degree of acetylation (DA), as well as its pattern of acetylation (PA) (Naqvi et al., 2016; Naveed et al., 2019; Bonin et al., 2020; Miao et al., 2021). Previous studies have demonstrated the antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activities of chitosan and its derivatives, highlighting their potential applications in the fields of food, medicine, and cosmetics (Ouyang et al., 2017; Lin et al., 2022; Sun et al., 2022; Chen et al., 2023).

Despite the considerable potential of chitin, its deacetylation process presents several challenges. Chitin is known for its insolubility in water and most organic solvents, which makes achieving uniform deacetylation difficult. Traditionally, chemical hydrolysis has been utilized for preparing chitosan and COS, but this method lacks control

over the structural properties of the products, involves the use of large quantities of strong acids and bases and is not environmentally friendly (Lodhi et al., 2014; Naveed et al., 2019; Arnold et al., 2020). In recent years, there has been an increased focus on the enzymatic deacetylation of chitin (CDA), which offers precise control over the DD in chitosan and the production of specific PA (paCOS) products. Additionally, enzymatic deacetylation is considered gentler, greener, more efficient, and more sustainable (Schmitz et al., 2019; Charoenpol et al., 2023; Cheng et al., 2023; Zhang et al., 2023).

The currently studied CDA enzymes are increasingly recognized as valuable tools for the production of fully defined paCOSs due to their selectivity in targeting specific regions. However, there is a lack of research on CDA production by strains of the genus *Rhodococcus* (Aragunde et al., 2018; Grifoll-Romero et al., 2018; Bai et al., 2020; Chai et al., 2020; Cheng et al., 2023). Previous studies have suggested that *Rhodococcus* spp. have the potential to produce highly active CDA. However, there are a limited number of isolated and characterized model members of the genus *Rhodococcus* from the environment, with only 97 known species. Furthermore, there are few reports on chitin deacetylation activity among these model species.

Currently, chitinase and chitosanase are commercially available, but chitin deacetylase remains unavailable. Numerous chitin deacetylases from various sources, including bacteria, fungi, and insects, have been identified and characterized, with particular attention given to their structure, mechanism of action, and substrate specificity. Studies have demonstrated that CDAs from different species exhibit diverse biological activities, with variations in their substrates and reaction conditions (Aragunde et al., 2018). Although recent research has reported on CDA enzyme activity in chitin deacetylation by strains from several genera, such as *Acinetobacter* (Yang et al., 2022a, 2023), *Bacillus* (Liang et al., 2022; Zhang et al., 2023), *Arthrobacter* (Ding et al., 2021), *Microbacterium* (Yang et al., 2022a) and *Streptomyce* (Yin et al., 2022), these CDA activities are either low or confined to narrow substrate specificities against chitin or CTO. Consequently, numerous studies have been conducted to optimize the reaction conditions, aiming to enhance the efficiency and yield of enzymatic deacetylation (Sun et al., 2014; Yang et al., 2022a).

The currently studied CDA enzymes are increasingly recognized as valuable tools for the production of fully defined paCOSs due to their selectivity in targeting specific regions (Aragunde et al., 2018; Bonin et al., 2020; Biniek-Antosiak et al., 2022; Cheng et al., 2023). However, there is a lack of research on CDA production by strains of the genus *Rhodococcus*. Previous studies have suggested that *Rhodococcus* spp. have the potential to produce highly active CDA (Sun et al., 2014; Ma et al., 2020). However, there are a limited number of isolated and characterized model members of the genus Rhodococcus from the environment, with only 97 known species. Furthermore, there are few reports on chitin deacetylation activity among these model species.[1]

In contrast, the currently available *Rhodococcus erythropolis* strain HG05, which has undergone complex fermentation optimization, only exhibited an enzyme activity of 238.89 U/mL (Sun et al., 2014). In this study, we identified a CDA-producing *Rhodococcus* strain, T22.7.1, which is suspected to be a new species. We investigated its taxonomic status using various methods and optimized the conditions for the production of its enzymes. Through simple process optimization, we achieved a much higher enzyme activity than what has been reported for existing *R. erythropolis* strains. We further isolated and obtained the purified CDA natural enzyme and investigated its enzymatic properties. This allowed us to determine the broad range of substrates it acts on and the characteristics of its catalytic products. Our findings demonstrate that strain T22.7.1 is a more efficient and highly active CDA-producing strain for the deacetylation of chitin and CTO. Additionally, we used genome mining and product characterization to clarify the functional potential of the Rhodococcus strain T22.7.1 in chitin biotransformation. Our study provides a valuable enzymatic tool for the environmentally friendly and efficient production of biologically active paCOS.

Materials and methods

Chemicals and reagents

Chitin was purchased from Aladdin Biochemical Technology Co. Ltd. (Shanghai, China); chitosan and chitosan oligosaccharides were purchased from Yuanye Biotechnology (Shanghai, China); N-acetyl-D-glucosamine was purchased from Solebo (Beijing, China). The colloidal chitin was prepared as the previously reported method (Pareek et al., 2013). p-Nitroacetanilide was purchased from Bailingwei Technology Co., Ltd. (Beijing, China). Bacterial genomic DNA extraction kit and PCR product/gel extraction kit were purchased from Tigen Biochemicals (Beijing, China); acetic acid assay kit was purchased from Solepol (Beijing, China); Millex®-GP 0.22um disposable injectable membranes and Ultra-15 centrifugal filters were purchased from Merck-Millipore (Beijing, China). Superdex 75 Increase 10/300 GL and Q Sepharose High Performance were both purchased from GE Healthcare (Sweden). Molecular weight markers (14.4 to 116 kDa) were purchased from Sangon Biotech Co. N-acetyl chito-oligomers ($(GlcNAc)_{1-6}$) were purchased from Qingdao BZ Oligo Biotech Co. The chito-oligomers mixture was purchased from TCI (Shanghai, China). All other chemicals and reagents were analytically pure.

Strain isolation and preservation

The strain T22.7.1^T with obvious deacetylation ability was isolated from the rhizosphere of *Acanthus ebracteatus* of Guangxi Beihai beach (N21.624, E108.909), China. The strain was purified by dilution plating method and streak inoculated on chitin deacetylation functional screening medium (g/L: p-nitroacetanilide 2; colloidal chitin 2; K_2HPO_4 0.7; KH_2PO_4 0.3; $MgSO_4$ 0.5; NaCl 0.1; agar 20; pH 7.0) (Zhang et al., 2023). Strain T22.7.1^T was inoculated by streaking on ISP2 medium and collected after 72 h of purification incubation at 28°C, and stored in 30% (v/v) glycerol at −80°C (Xie et al., 2023a). The strain has been deposited in the Marine Culture Collection of China (MCCC) and Japan Microbe Division (JCM) under the following numbers: MCCC 1 K08698 and JCM 36625, respectively.

1 https://lpsn.dsmz.de/

Extraction, sequencing and phylogenetic analysis of 16S rDNA gene and genome

The genome of strain T22.7.1^T was extracted using the Genomic DNA Extraction Kit (TIANGEN) and the 16S rRNA gene was amplified as previously described by Li *et al* (Li et al., 2007). 16S rRNA gene sequencing was performed by Wuhan AOKE DINGSHENG BIOTECHNOLOGY CO., LTD.[2] using the Sanger method. The 16S rDNA sequence of strain T22.7.1^T was identified and analyzed using BLAST[3] and EzBioCloud[4] server databases, and online comparison analysis was performed to obtain the 16S rDNA sequences with the highest degree of similarity to the strain. The 16S rDNA sequences of strain T22.7.1^T and its close relatives were analyzed for multiple comparisons and sequence similarity levels were calculated using the Clustal W program of MEGA 11 (Tamura et al., 2021); neighbor-joining (Saitou and Nei, 1987), maximum likelihood (Felsenstein, 1981), and maximum parsimony (Fitch, 1971) were used to construct a phylogenetic tree using a self-expansion value of 1,000 resampling replicates, respectively (Felsenstein, 1985). The whole genome draft of strain T22.7.1^T was sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.[5] using Illumina HiSeq X10 platform. The complete genome sequence was assembled *de novo* by SPAdes 3.13.0 using the online patric server (Bankevich et al., 2012; Wattam et al., 2017). The whole genome sequence of strain T22.7.1^T was phylogenetically analyzed using the Type Strain Genome Server (https://tygs.dsmz.de) (Meier-Kolthoff and Göker, 2019), and by the genome blast distance phylogeny (GBDP) based FastME 2.1.6.1. program to construct a phylogenetic tree (Lefort et al., 2015).

The complete 16S rDNA sequence (1,473 bp) and the whole genome sequence (WGS) of strain T22.7.1^T were submitted to the GenBank database with the DDBJ/EMBL/GenBank accession numbers OQ976993 and JASKMB000000000, respectively.

Culture and phenotypic characterization

Cell morphology was visualized by field emission scanning electron microscopy (Carl Zeiss Supra 55 Sapphire) after 2 weeks of incubation at 28°C on ISP 2 agar. Culture characteristics were examined after 2 weeks of incubation at 28°C on ISP 1–7 agar (Shirling and Gottlieb, 1966), Czapek agar (CA) (Waksman, 1967), tryptic soy agar (TSA; Difco), starch ammonia agar (SAA) (Barreiro et al., 2021), and lysogeny broth agar (LB, Difco). Colony color was determined according to the ISCC-NBS color scale (No. 2106) (Kelly, 1964). Cell motility was determined according to the previous method (Leifson, 1960). Temperature (10–60°C at 5°C intervals), pH [4–11 at 1 interval, buffer systems used refer to previous descriptions (Xie et al., 2023a)] and NaCl (0–14% w/v at 2% intervals) tolerance assays were tested on ISP 2 medium, incubated at 28°C for 2 weeks each and continuously monitored (Ramaprasad et al., 2015; Maiti and Mandal, 2019; Maiti and Mandal, 2020).

Chemical taxonomic analysis

Biomass for chemical composition analysis was obtained and lyophilized as the methods described by us previously (Xie et al., 2023a,b)Whole cell hydrolysate sugars and characteristic amino acids of strain T22.7.1^T were analyzed by thin layer chromatography (TLC) (Lechevalier et al., 1971; Staneck and Roberts, 1974). Extraction of polar lipids and methylnaphthoquinone was carried out following the method described by Minnikin et al. (1984), and the polar lipids were analyzed by spreading according to the two-dimensional TLC method described by Goodfellow et al. (1980). The main respiratory quinone types of strain T22.7.1^T were analyzed by HPLC method (Kroppenstedt, 1982). The cellular fatty acid composition was tested by the Marine Conservation Center of China (Xiamen, PR China) using the Sherlock Microbial Identification System, and the fatty acid methyl esters were quantified using the TSBA 6.0 database (Sasser, 1990).

Comparative genomic analysis

Comparative analysis of the genomic data of strain T22.7.1^T and the species most relevant to its development was performed based on phylogenetic relationships of whole genome sequence reconstruction. Genomic coding sequences (CDSs) of the strain genome were annotated using the Rapid Annotation Tool of the Subsystems Technology server (RAST) (Aziz et al., 2008). Comparative analysis of homology clusters of strain T22.7.1^T and its close relatives was performed using OrthoVenn3 (Sun et al., 2023). The average nucleotide identity (ANI) value between strain T22.7.1^T and its closer relatives was calculated using JSpecies Web Server based on the Blast+ method (Richter et al., 2016). Digital DNA–DNA hybridization (dDDH) values based on the strain's whole genome sequence, Genome-to-Genome Distance and Difference %G + C were calculated using the Genome-to-Genome Distance Calculator (GGDC 3.0; http://ggdc.dsmz.de) and the recommended dDDH results of Equation 2 were adopted (Meier-Kolthoff et al., 2022).

Genome mining and metabolic system analysis

Coding sequences (CDS) in the genome were predicted using Prodigal, which was used to predict chromosomal genes, and GeneMarkS, which was used to predict plasmid genes. The tRNAs and rRNAs contained in the genome were predicted using tRNAscan-SE[6] and Barrnap,[7] respectively. The predicted CDSs were annotated using Non-Redundant Protein Database (NR), Swiss-Prot (Bairoch and Apweiler, 2000), Pfam (Mistry et al., 2021), evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (Jensen et al., 2008), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) databases using Blast2Go, Diamond and HMMER3 as sequence alignment tools (Sun

2 http://wh.augct.com/
3 https://blast.ncbi.nlm.nih.gov/Blast.cgi
4 www.ezbiocloud.net
5 https://www.majorbio.com
6 http://trna.ucsc.edu/software/
7 https://github.com/tseemann/barrnap

et al., 2016; Xiao et al., 2022). Briefly, the query proteins were aligned with the databases to obtain the gene annotations corresponding to the most optimal matches (E-value $<10^{-5}$).

The metabolic system analysis of the strains included annotation of carbohydrate-activating enzymes and secondary metabolite synthesis gene cluster analysis. HMMER3 was used to derive annotation information corresponding to carbohydrate-active enzymes (CAZymes) based on whole-genome sequence deduced to protein sequence comparison to the CAZymes database (CAZy, http://www.cazy.org/) (Drula et al., 2022). The screening condition was E-value $<1e^{-5}$. On the other hand, the strain genomes were rapidly identified, annotated and analyzed using antiSMASH 7.0 to know the functional potential of microorganisms to synthesize secondary metabolites (Blin et al., 2023).

Optimization of fermentation conditions

Strain T22.7.1 was aseptically inoculated into ISP2 liquid medium fermented at 28°C, 180 rpm, and shaking flask for 48 h as seed solution. The fermented seed liquid was inoculated into a sterilized 30 mL basal fermentation medium at a 1% (v/v) inoculum volume. It was then placed in a shaker at 28°C and 200 rpm for 72 h to cultivate the CDA enzyme through liquid fermentation under basal conditions.

The fermented seed liquid was inoculated into the sterilized 30 mL (20% loading, v/v) basal fermentation medium (g/L, KH_2PO_4 0.3, K_2HPO_4 0.7, $(NH_4)_2SO_4$ 2, glucose 2.5, yeast extract 5) at 1% (v/v) inoculum volume. It was then placed in a shaker at 28°C and 200 rpm for 72 h to cultivate the CDA enzyme through liquid fermentation under basal conditions. To optimize the medium composition and culture conditions, both a one-way experiment and an orthogonal design were employed. The one-way experiments focused on optimizing one ingredient at a time in the basic fermentation medium. Each condition was optimized based on the previous one. Several carbon sources (chitin, glucose, maltose, fructose, sucrose, and soluble starch), nitrogen sources (yeast extract, beef paste, peptone, soybean extract, ammonium chloride, and urea), and inorganic salts ($CaCl_2$, $MnSO_4$, $FeSO_4$, KCl, NaCl, and $MgSO_4$) were selected as candidate components for the medium composition. The basal medium was used as a control for optimizing the medium composition. Furthermore, the initial pH of the medium and fermentation conditions (temperature, fermentation time, loading volume, and inoculum volume) were optimized in a similar manner.

CDA enzyme activity assay

The colorimetric method (p-nitroacetanilide as substrate) used to assay CDA enzyme activity was slightly modified from a previous description (Liping, 2008). The standard curve was obtained by determining the correspondence between the absorbance value of the reaction substrate p-nitroacetanilide deacetylation product p-nitroaniline at 400 nm, OD_{400}, and the concentration of the product. In each numbered 1 mL capacity eppendorf centrifuge tube (EP tube), 0.3 mL of phosphate buffer solution at pH 7 concentration of 0.05 mol/L was added, and after a water bath at 50°C for 5 min, 0.1 mL of p-nitroacetanilide solution at concentration of 200 mg/L and 0.1 mL of crude enzyme solution were added respectively, and 0.1 mL of the control colorimetric tube was added. Add 0.1 mL of p-nitroacetanilide solution, 0.1 mL of crude enzyme solution after inactivation in boiling water bath, add the crude enzyme solution after inactivation in boiling water bath; keep it at 50°C for 15 min, then put it into boiling water bath for 5 min, cool it down, and then make it into 1 mL with distilled water, shake it well and centrifugate at 10000 rpm for 10 min, and then take supernatant to determine the absorbance value at 400 nm wavelength. The enzyme activity was calculated against the standard curve of p-nitroaniline.

The enzyme activity unit (U) of CDA was defined as the amount of enzyme required to catalyse the production of 1 μg of p-nitroaniline from the substrate by chitin deacetylase per hour under the above conditions (Sun et al., 2014). The formula for the standard curve used to calculate the p-nitroaniline content of the product, constructed according to the above method, is given below:

$$y = 0.1047x + 0.0457 \left(R^2 = 0.9999\right)$$

HPLC method used to the different deacetylation activities of strain CDA on chitin, chitosan, CTO, CSO, and NAG were tested by using an acetic acid content assay kit and by high-performance liquid chromatography (HPLC) detection technique (Bai et al., 2020; Cheng et al., 2023). The monosaccharide and oligosaccharide substrates were dissolved in phosphate buffer (50 mM, pH 7.0) and 500 μL of substrate solution was taken and 500 μL of enzyme solution was configured to form a 1 mL reaction system, which was placed in a 50°C water bath for 1 h and then boiled for 10 min to terminate the reaction. Insoluble polysaccharide substrate with enzyme solution and phosphate buffer solution to form a 1 mL reaction system was placed in 37°C shaker 180 rpm shaking 3 h after boiling for 10 min to terminate the reaction. The reaction mixture was centrifuged at 10000 rpm for 20 min, and the supernatant was filtered through a 0.22 μm membrane and used as the sample for HPLC analysis under the following conditions: detection on an Ultimate AQ-C18 column (5 μm, 250 × 4.6 mm) at a column temperature of 30°C and a UV filter at a wavelength of 210 nm, and the flow rate was 0.4 mL/min. Each acetic acid detection was repeated three times. The concentration of acetic acid in the samples was calculated using standard curves generated from 175, 87.5, 17.5, 8.75, 1.75 and 0.875 μmol/mL of acetic acid with the following equation:

$$y = 40.557x - 31.508 \left(R^2 = 0.9998\right)$$

The amount of enzyme required to release 1 μmol of acetic acid per minute under the above reaction conditions was defined as one unit of enzyme activity (U) (Bai et al., 2020).

Enzyme purification, enzymatic properties and product characterization

The purification of CDA was carried out with slight modification of the method described by a previous author (Chai et al., 2020). The strain was cultured in the optimized fermentation medium and conditions to produce the CDA enzyme. The fermentation product was subjected to ultrasonic cell disruption in an ice bath, followed by centrifugation at 12000 rpm for 20 min. The resulting supernatant was

considered the crude enzyme broth. Ammonium sulfate powder was added to the crude enzyme broth in an ice bath until a saturation level of 75% was reached. The precipitate was collected by centrifugation at 12000 rpm for 20 min and reconstituted with ultrapure water; residual ammonium sulphate was removed and replaced with 10 mM phosphate buffer (pH 7.0) using a 30 kDa ultrafiltration tube. The crude enzyme solution was loaded onto a Q Sepharose High Performance gel chromatography column (16 mm × 40 cm), pre-equilibrated with 10 mM sodium phosphate buffer pH 7.0, and then eluted with a linear gradient of 0–1.0 M NaCl. The eluted components were UV-detected at 280 nm as described above, and the eluted peak components were collected into different EP tubes in 1 mL volumes. The enzyme activity in each collection tube was tested using a colorimetric method, and based on the test results, the eluent with enzyme activity was mixed and introduced onto a Superdex 75 Increase 10/300 GL column (10 mm × 30 cm). The column was eluted with 50 mM Tris–HCl (pH 7.5) containing 0.15 M NaCl, and the elution components were monitored at 280 nm wavelength, and the elution peak components were collected into different EP tubes in 0.5 mL volumes. After CDA enzyme activity was tested by colorimetric method, the collected solution with CDA activity was mixed and replaced with a solution of 10 mM sodium phosphate buffer through a 30 kDa ultrafiltration tube. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% w/v polyacrylamide gel (Pareek et al., 2013). Proteins in the polyacrylamide gel were color developed with Coomassie Brilliant Blue G250.

Enzymatic property test

Enzyme activity was determined at temperatures ranging from 30 to 70°C (at 10°C intervals) using a phosphate buffer solution at pH 7.0. The reaction solution was maintained at the specified temperatures for 0–10 h, and enzyme activity was measured every 2 h. Enzyme activity at 0 h at each temperature was taken as 100% to study temperature stability. Different pH values were tested at 50°C in a water bath (pH 5–9 at intervals of 1, 50 mM citrate buffer 5–6, 50 mM phosphate buffer 6–8 and Tris- HCl buffer 8–9.) to assess CDA enzyme activity and stability. Metal salts (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Na^{+}, K^{+}) and chemical reagents (EDTA, SDS, PSMF, Tween 20, Tween 80) with the same ionic concentration were added to the reaction system to reach the final concentration of 1 mM for each metal ion and 5 mM for each chemical reagent, respectively and enzyme activity assay was carried out without adding any other substance to the reaction system as a control, and three replicates were set up for each set of experiments.

Characterization of the deacetylation products of different substrates

The deacetylation products of *Ri*CDA-treated CTO were analysed using the TLC method (Kang et al., 2014; Yang et al., 2022a). The reaction products were analysed with a TLC silica gel plate (Merck KGaA, Darmstadt, Germany), and the solvent for chromatography development was n-butanol/anhydrous ethanol/water (5,3,2, v/v). The spots of sugar on the plate were observed by spraying the color developer (1.0 g of diphenylamine dissolved in 50.0 mL of acetone, followed by the gradual addition of 1.0 mL of aniline and 5.0 mL of phosphoric acid) and baking the plate for 5 min at 85°C. On the other hand, chitin oligosaccharides (DP 1–5, TCI) were separated by TLC method, the color-developed spots were used as templates to scrape the silica gel at the position of specific shift values, and the scraped silica gel was dissolved in 1 mL of ultrapure water, mixed and filtered through a 0.22 μm membrane, and then the aqueous phase after extraction was repeated twice using methylene chloride to collect the aqueous phase after extraction as a single polymerization degree of the chitin oligosaccharides samples were used as substrates for subsequent *Ri*CDA treatment. SEM and FTIR of chitin deacetylation products: 0.2 g of chitin powder and 1 mL of *Ri*CDA enzyme solution were added to a 2 mL EP tube, mixed, and then placed at 37°C, 180 rpm shaker reaction for 12 h, boiled for 10 min to terminate the reaction, and centrifuged at 10000 rpm for 10 min, and then the precipitate was washed with deionized water for two times, and then dried at 50°C for 24 h as the samples for SEM and FTIR tests. SEM and FT-IR tests were performed using water-treated chitin powder as a control. Photographs of the surface microforms of the samples were taken using a Nippon Electron JSM-IT100 scanning electron microscope with a magnification of 2000, 5,000, and 10,000, respectively, following the previous methods (Li et al., 2019; Yang et al., 2022a). Dried potassium bromide and the samples were weighed according to the ratio of 120:1, and after grinding and mixing, 0.2 g of mixture was weighed and made into thin slices by using a hand-operated tableting press (769YP -15A) to make thin slices and FT-IR spectroscopy (Nicolet-iS10) was used to scan the region with a wave number of 400–4,000 cm^{-1}. The intensity ratio of the characteristic absorption peaks with wave numbers of 1,655 cm^{-1} (amide I) and 3,450 cm^{-1} (OH group) correlated linearly with the degree of deacetylation of the chitin samples, which was calculated using the following equation (Peh et al., 2000).

$$D.D = \left(1 - \frac{A_{1655} / A_{3450}}{1.33}\right) \times 100\%$$

Data analysis and graphing software

All trials were conducted in triplicate and data are expressed as mean ± standard deviation. One-way ANOVA and Duncan's multiple range test were performed using SPSS software (version 27.0) to test for variance and significant differences.

Results and discussion

Strain screening and identification

The results of 16S rDNA sequence of strain T22.7.1^{T} compared in Ezbiocloud database showed that strain T22.7.1^{T} was similar to *Rhodococcus ruber* DSM 43338^{T} (98.96%), *Rhodococcus electrodiphilus* JC435^{T} (98.93%) and *Rhodococcus aetherivorans* 10bc312^{T} (98.90%) had the highest similarity, while the rest of the strains of the genus *Rhodococcus* had similarities below 97.80%. The NCBI database comparisons showed similar results, with strain T22.7.1^{T} being similar to *R. ruber* DSM 43338^{T}, *R. electrodiphilus* JC435^{T}, and *R. aetherivorans* 10bc312^{T} with similarities of 98.97, 98.81, and 98.83%, respectively, and additional comparisons found that strain T22.7.1^{T} was similar to the most recently reported (January 2024) *R. indonesiensis* CSLK01-03^{T} with the highest similarity of 99.79%.

The phylogenetic tree constructed using the aligned 16S rDNA sequences (full length 1,473 bp) showed that strain T22.7.1^T was most closely related to the strain *R. indonesiensis* CSLK01-03^T, as well as to *R. ruber* DSM 43338^T, *R. electrodiphilus* JC435^T, and *R. aetherivorans* 10bc312^T. *R. indonesiensis* CSLK01-03^T clustered as a branch of the evolutionary tree (Figure 1), consistent with the phylogenetic tree constructed based on the whole-genome sequence of the strain (Figure 2), verifying that the strain is a member of the genus *Rhodococcus*.

And based on the whole genome sequence comparison analysis, it was found that the genome-to-genome distance between strain T22.7.1^T and strains *R. indonesiensis* CSLK01-03^T, *R. ruber* DSM 43338^T, *R. electrodiphilus* JC435^T, and *R. aetherivorans* 10bc312^T were 0.0101, 0.0489, 0.0493 and 0.0882, respectively, from which the evolutionary relationship between the strains and their close relatives could be inferred.

The culture morphology of strain T22.7.1^T on screening plate media and LB agar media is shown in Figure 3A and

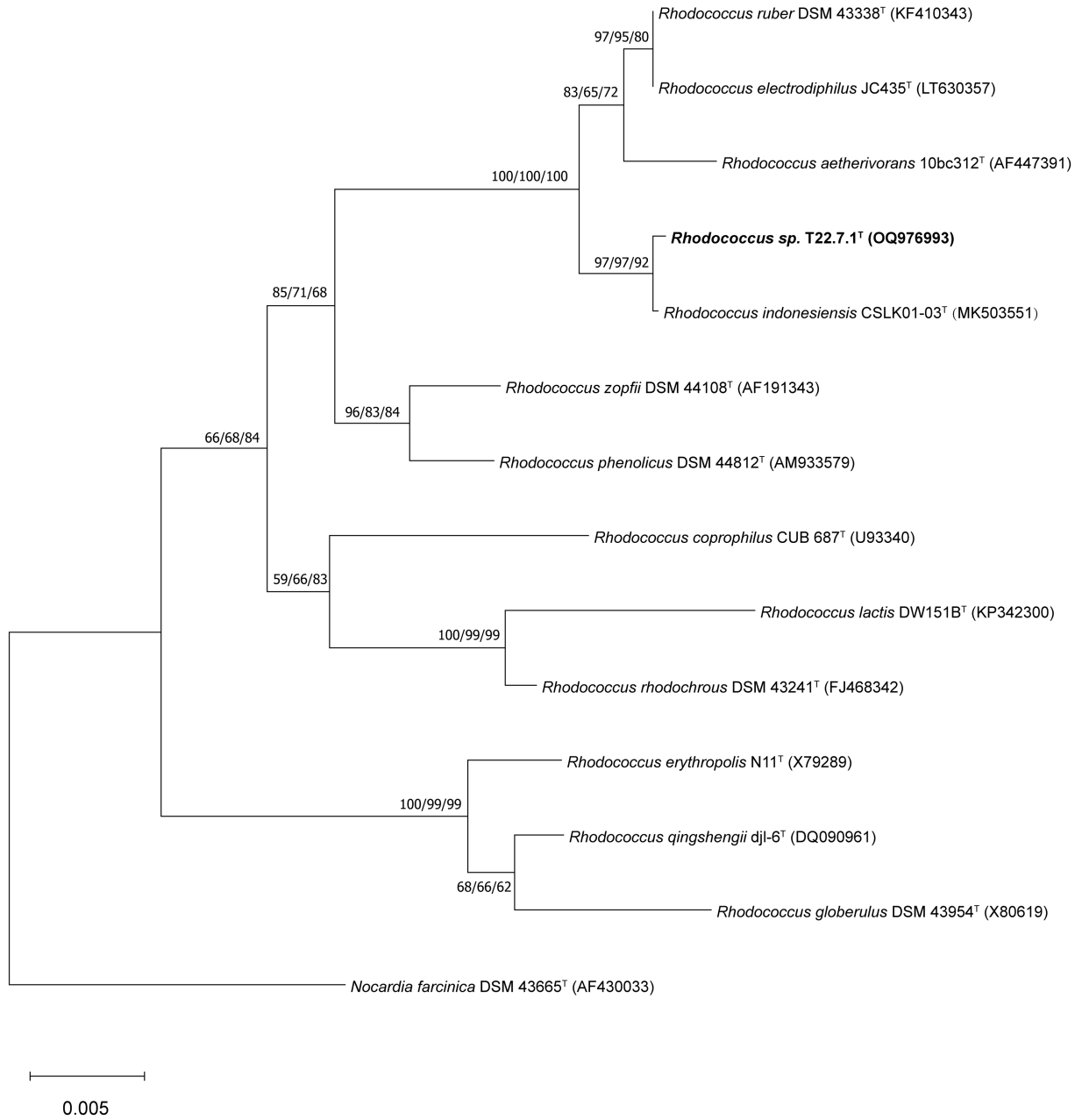


FIGURE 1
Phylogenetic tree based on 16S rDNA sequences showing the relationship of strain T22.7.1^T to the most closely related members of the genus *Rhodococcus*. The tree was constructed using the NJ method and the phylogenetic tree was reconstructed using the ML and MP methods, with the number before each branch node representing the bootstrap value and the percentage of bootstrap involved in the NJ/ML/MP analysis, and the numbers below 50% percentage of bootstrap were omitted. *Nocardia farcinica* DSM 43665^T was defined as an outgroup of the tree and used as the root. The GenBank accession number for the 16S rDNA sequence of each strain is listed in parentheses. The scale bar indicates 0.005 substitutions per nucleotide position.

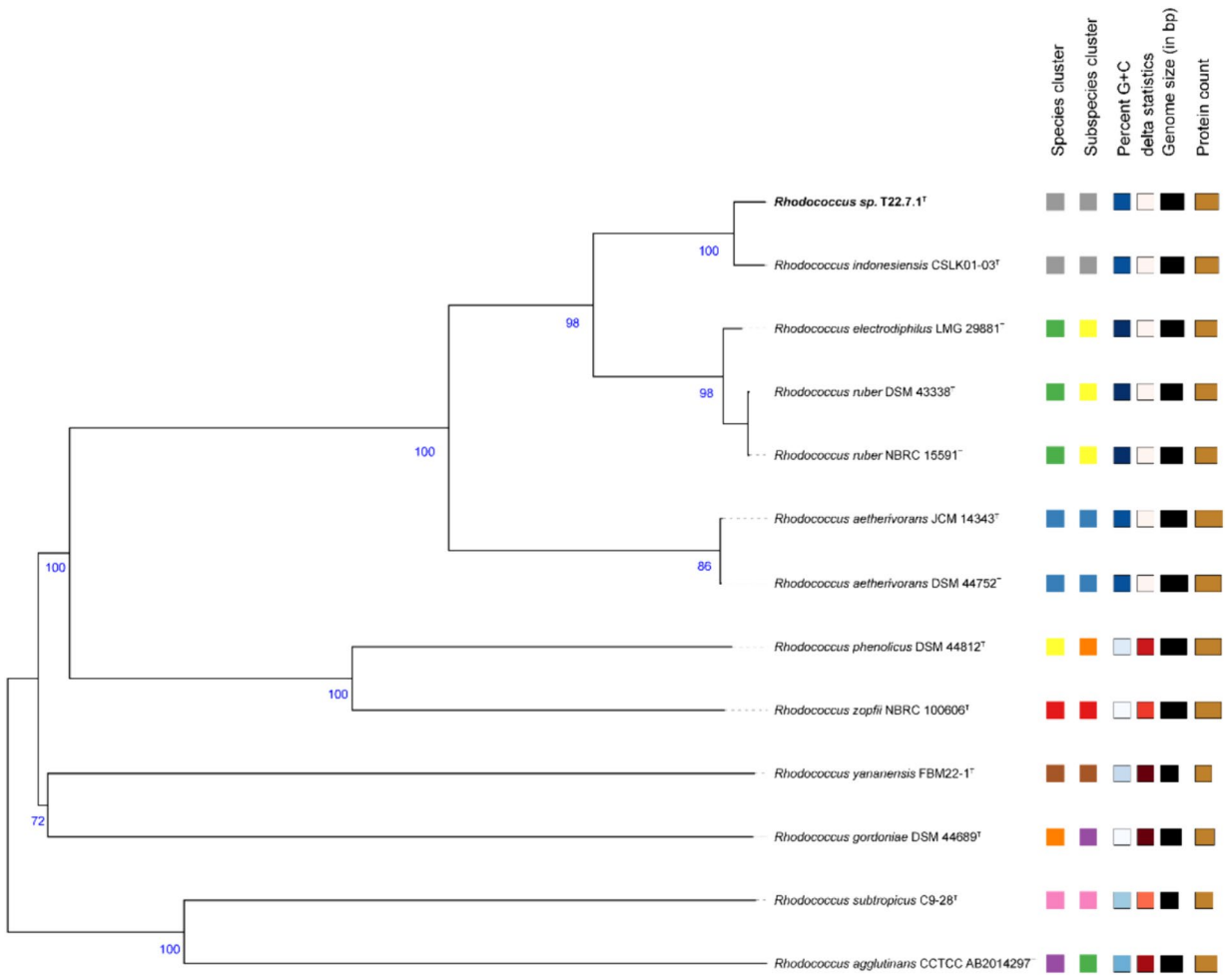


FIGURE 2
Phylogenetic tree based on whole-genome sequences. This tree is inferred with FastME 2.1.6.1 in the TYGS website from genome blast distance phylogeny (GBDP) approach. Branch length is scaled by GBDP distance formula d5. The number of branches above is 100 replicates of GBDP pseudo-guidance support value >60%, and the average branch support is 90.9%. The tree was rooted at the midpoint.

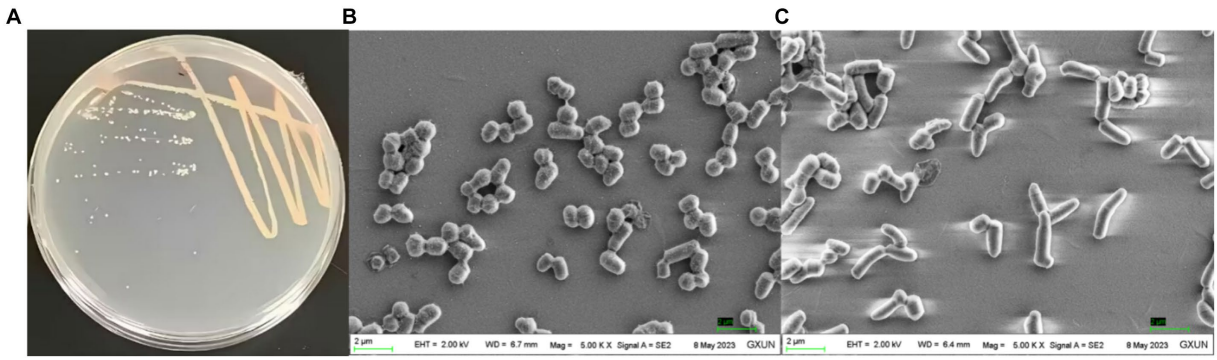


FIGURE 3
Culture morphology **(A)** and cell morphology **(B,C)** under scanning electron microscopy of the newly isolated strain T22.7.1^T. **(A)** shows the strain cultured on LB medium for 2days when the color of the strain started to change from light yellow-white to orange-red; **(B)** shows the cells of the strain appearing in the shape of a sphere with a magnification of 5,000; **(C)** shows the cells of the strain appearing in the shape of a rod with a magnification of 5,000.

Supplementary Figure S1A. The purified strain T22.7.1^T showed a single colony of nondiffuse growth on the screening medium, with a light yellow–white color. Prolonged incubation led to the deacetylation of p-nitroacetanilide in the light-yellow medium, resulting in a brighter golden yellow color (Supplementary Figure S1A). This indicates that the strain can produce chitin deacetylase, making it a target strain for

studying chitin/CTO deacetylation (Liping, 2008). Compared with screening media supplemented with colloidal chitin as the sole carbon source, LB media (after 2 days of incubation the growth was denser, and the strain began to shift from a light yellowish-white color to an orange-red coloration, whereas it was slow-growing and did not produce any red pigmentation on the screening medium) provided more favourable growth conditions for strain T22.7.1^T, as evidenced by its faster growth rate on LB media. Morphological characteristics of strain T22.7.1^T on other media are recorded in Supplementary Table S1.

The cell morphology of strain T22.7.1^T was short rod-shaped (1.8–3.2 μm) to globular (1.2–1.6 μm) (Figures 3B,C), a characterization that is consistent with that of its close species relatives, strains *R. indonesiensis* CSLK01-03^T, *R. electrodiphilus* JC435^T, *R. ruber* DSM 43338^T, and *R. aetherivorans* 10bc312^T, but the cell size of strain T22.7.1^T is slightly longer (Ramaprasad et al., 2018; Kusuma et al., n.d.). Additionally, at 100,000× magnification, two distinct rings of annular protrusions were observed at the ends of the cells, along with terminal bulbous protrusions forming a cap-like structure (Supplementary Figure S1B), a feature not previously reported for closely related species of strain T22.7.1^T. The cell morphology of strain T22.7.1^T exhibited two or more cells side-by-side or in tandem, differing markedly from that of its close relatives: *R. electrodiphilus* JC435^T, *R. ruber* DSM 43338^T, and *R. aetherivorans* 10bc312^T; strain *R. electrodiphilus* JC435^T cells arranged in rows of 4–5 cells; *R. ruber* KCTC 9806^T cells mostly forming tetrads; and *R. aetherivorans* JCM 14343^T cells mostly bundled (Ramaprasad et al., 2018). Additionally, strain T22.7.1^T proved to be an aerobic, Gram-stain-positive, nonmotile strain that was able to tolerate up to 12% (w/v) NaCl (optimal concentration 1% (w/v)), grow in a pH range of 5–9 (optimal pH 8), and thrive at temperatures ranging from 10 to 45°C (optimum 35°C). More morphological and cultural characteristics as well as physiological and biochemical characteristics are detailed in Table 1 and Supplementary Table S2. The morphological, biochemical, and physiological characteristics of strain T22.7.1^T conform to the typical characteristics of *Rhodococcus* strains (Parte et al., 2012; Lee et al., 2020; Sun et al., 2023).

Chemical taxonomic characterization

The whole-cell sugars of strain T22.7.1^T were identified as glucose and galactose. The characteristic diamino acid, meso-diaminoheptanedioic acid, was detected in the cell wall hydrolysate of the new isolate. The major fatty acid fractions (>10%) of strain T22.7.1^T were $C_{16:1}$ ω6c/$C_{16:1}$ ω7c (10.76%), $C_{16:0}$ (26.81%), and $C_{18:0}$ 10-methyl-tuberculostearic acid (TBSA) (14.97%), and the detailed distribution of fatty acid content is shown in Supplementary Table S3. In the strain T22.7.1^T, 12 lipids were detected, which were diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylinositol, phosphatidylglycerides phosphatidylinositol mannosides, phosphatidylinositol dimannosides, two unknown glycolipids, two phospholipids of unknown structure containing glucosamine and two unknown lipids (Supplementary Figure S2). The predominant respiratory quinone type (>10%) of strain T22.7.1^T was MK-8(H2) (96.3%), with small amounts of MK-7(H2) (2.3%) and MK-8 (1.4%) also detected. All the chemotaxonomic features of strain T22.7.1^T were consistent with the genus *Rhodococcus* (Parte et al., 2012; Lee et al., 2020; Zhang et al., 2021; Sun et al., 2023). Comparison of chemical taxonomic characters between strain T22.7.1^T and its close relatives is shown in Table 1.

Comparative genomic analysis

The core and specific genes of strain T22.7.1^T and the strains most relevant to its development were determined using OrthoVenn3 analysis. A total of 4,198 core gene clusters were identified in the five strains (Figure 4), of which six genes were specific to T22.7.1^T, which encodes functional small molecules such as deconjugating enzymes, hydrogen sulfide hydrolysis, and nitrogen-containing compound hydrolysis activities. A comparison of the number of homologous genes revealed that T22.7.1^T had the highest similarity 90.93% (5,102/5611) with *R. indonesiensis* CSLK01-03^T. However, *R. electrodiphilus* JC435^T (84.70%), *R. ruber* DSM 43338^T (84.42%), *R. aetherivorans* 10bc312^T (74.49%) showed low sequence similarity. In addition, the ANI and dDDH values based on whole-genome sequences showed that strains T22.7.1^T and *R. indonesiensis* CSLK01-03^T had the highest similarity (98.90/91.90), exceeding the critical values for the delineation of the same species (ANI value >95%, dDDH value >70%), whereas strains T22.7.1^T and *R. aetherivorans* 10bc312^T (74.49%) had lower sequence similarity (Table 2). Although strain T22.7.1^T had ANI and dDDH values lower than the critical values for three other closely related strains, strain T22.7.1^T can be recognized as another type of strain under the species classification of *R. indonesiensis* (Goris et al., 2007; Richter and Rossello-Mora, 2009). In this study, we found that the ANI and dDDH values (98.76/92.50) between *R. ruber* DSM 43338^T and *R. electrodiphilus* JC435^T also exceeded the critical values for species classification, validating recent findings by Kusuma et al. to recognize *R. ruber* DSM 43338^T and *R. electrodiphilus* JC435^T as the same species (Kusuma et al., n.d.). These results help to isolate strain T22.7.1^T from the closely related type strain and support the determination of its taxonomic status.

Genome characterization, annotation and functional prediction:

The draft genome of strain T22.7.1^T consisted of 112 contigs with a total genome size of 5.53 Mbp and a G + C content of 70.17 mol%. These data are consistent with the characterization of draft genome sizes ranging from 3.9 to 10.4 Mbp and DNA G + C contents ranging from 61.8 to 70.7 mol% in the genus *Rhodococcus* (Nouioui et al., 2018; Zhang et al., 2021; Kusuma et al., n.d.). Based on the comparative analysis of genome size and G + C content, strain T22.7.1^T had a slightly larger genome (5.48 Mbp) and higher G + C content than *R. indonesiensis* CSLK01-03^T (Supplementary Table S5), indicating that strain T22.7.1^T is different from CSLK01-03^T. The number of protein-coding genes and RNA genes was 5,092 and 67, respectively, and more features of strain T22.7.1^T genome annotation, such as comparison of annotation results from databases such as NR, Swiss-Prot, Pfam, eggNOG, GO and KEGG, are listed in Supplementary Table S4.

Secondary metabolites are generally controlled by multiple genes, which usually exist in clusters in the genome and encode complex enzymes with multiple functions. These clusters are the secondary metabolite biosynthesis-related gene clusters (smBGCs). The smBGCs of strain T22.7.1^T and its close relatives were predicted by antiSMASH. A total of 24 putative smBGCs were detected in strain T22.7.1^T. The identified smBGCs showed homology to 14 gene clusters with known metabolites such as ε-poly-L-lysine, ectoine, heterobactin A/heterobactin S2, and isorenieratene. In addition, strain T22.7.1^T contains a unique gene cluster (Liu et al., 2014), the stenothricin synthesis gene cluster. The smBGCs of *R. indonesiensis* CSLK01-03^T,

TABLE 1 Differential characteristics between strain T22.7.1^T and its closely species strains.

Characteristics	1	2	3	4	5
Isolation source	Mudflat sediment	Hot spring sediment	Marine coral reef	Sediment	Petrochemical biotreater sludge
Colony colour	Orange red	Reddish orange	Dark red	Pale orange	Pinkish
Temperature range for growth (°C)	10–45	10–39	20–40	10–40	10–40
NaCl range for growth (%, w/v)	0–12	0–10	0–13	0–12	0–12
pH tolerance for growth	5.0–9.0	6.5–8.0	6.5–11.0	5.5–8.5	6.0–8.5
Hydrolysis of:					
Casein	–	–	+	–	+
Starch	–	–	+	+	+
Tween 20	–	–	–	+	–
Tween 60	–	–	+	+	–
Tween 80	–	–	+	+	–
Chitin	–	+	+	–	–
Urea	–	–	–	+	–
Xylan	–	+	+	–	+
Growth on sole carbon sources:					
myo-Inositol	+	+	+	–	–
Sodium Butyrate	–	–	+	+	–
Sodium Gluconate	+	+	–	+	–
$_D$-mannitol	+	–	+	–	+
$_D$-sorbitol	+	+	+	+	–
Maltose	+	+	–	+	+
N-Acetyl glucosamine	+	–	+	–	+
Cellobiose	+	+	+	–	–
$_D$-galactose	+	–	+	–	+
Cellulose	–	–	+	–	–
Sucrose	+	–	+	–	+
$_L$-rhamnose	+	–	+	–	–
Phospholipids[†]	DPG, PME, NPG, PG, PI, PLs, GLs, PIM, PIDM	DPG, PE, PME, PI, PIM, GLs, PLs	DPG, PE, PI, PIM, PLs, ULs, AL	DPG, PE, PI, PIM, PLs	DPG, PE, PI, PIM, PL
Major fatty acids	$C_{16:1}$ ω6c/ω7c, $C_{16:0}$, $C_{18:0}$ 10-methyl- TBSA	$C_{16:0}$, $C_{18:1}$ ω9c, $C_{18:0}$ 10-methyl-TBSA, $C_{18:2}$ ω6/anteiso-$C_{18:0}$ 9c	$C_{16:0}$, $C_{17:1}$ iso I/anteiso B, $C_{18:1}$ ω9c, $C_{18:0}$ 10-methyl-TBSA	$C_{16:1}$ ω6c/ω7c, $C_{16:0}$, $C_{18:1}$ ω9c, $C_{18:0}$ 10-methyl- TBSA	ND
Major menaquinone	MK-7(H2), MK-8, MK-8(H2)	MK-8, MK-8(H2)	MK-8(H2), MK-7(H2), MK-9(H2)	ND	ND

[†]DPG, diphosphatidylglycerol; PME, phosphatidylmethylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPG, lyso-phospatidyl glycerol; PGL, phosphatidylglycerides; PG, phosphatidylglycerol; PS, phosphatidylserine; PIM, phosphatidylinositol mannosides; PIDM, phosphatidylinositol dimannosides; NPG, phospholipids of unknown structure containing glucosamine; PLs, unknown phospholipids; GLs, unidentified glycolipid; ULs, unidentified lipids; ALs, unidentified aminolipids. Strains: 1, T22.7.1^T; 2, R. indonesiensis CSLK01–03^T [*data from Kusuma et al., n.d.]; 3, R. electrodiphilus JC435^T [*data from Ramaprasad et al., 2018]; 4, R. ruber DSM 43338^T [*data from Ramaprasad et al., 2018]; 5, R. aetherivorans 10bc312^T [*data from Ramaprasad et al. (2018)]. –, Negative; +, positive; w, weakly positive; ND, not determined.

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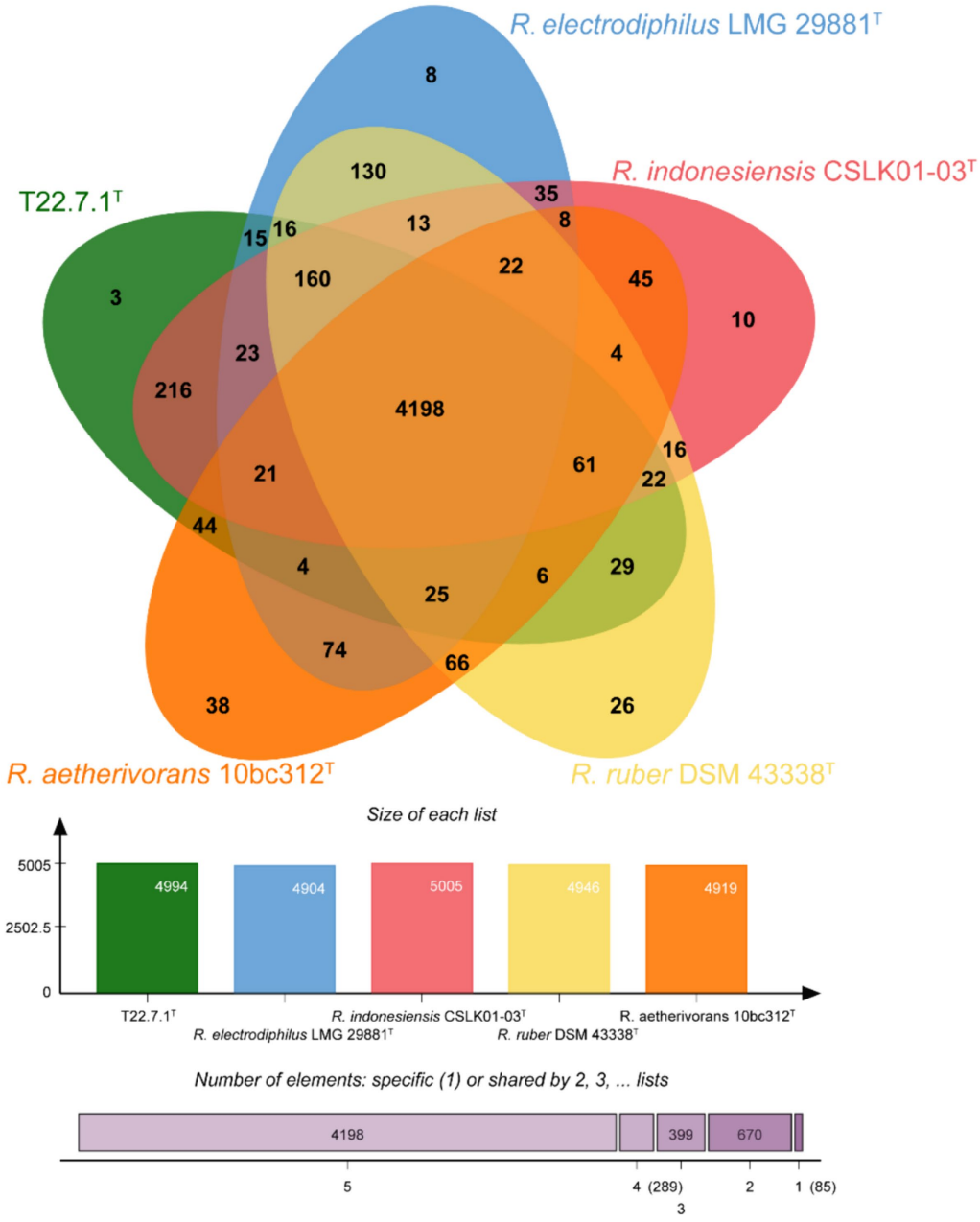

FIGURE 4
Venn diagram representing the core orthologs and unique genes for strain T22.7.1T and closely related type strains.

TABLE 2 ANI and dDDH values found between strain T22.7.1T and its closest related type strains.

ANI/dDDH (%)	T22.7.1T	*R. indonesiensis* CSLK01-03T	*R. ruber* DSM 43338T	*R. electrodiphilus* JC435T	*R. aetherivorans* 10bc312T
T22.7.1T	100/100				
R. indonesiensis CSLK01-03T	98.90/91.90	100/100			
R. ruber DSM 43338T	94.90/61.60	94.85/61.40	100/100		
R. electrodiphilus JC435T	94.69/61.40	94.67/61.30	98.76/92.50	100/100	
R. aetherivorans 10bc312T	89.97/43.60	89.95/43.60	90.34/44.90	90.14/44.60	100/100

ANI value above cutoff (>95%) is displayed in green, and dDDH value above cutoff (>70%) is displayed in blue when they are compared in different strains.

R. ruber DSM 43338^T, *R. electrodiphilus* JC435^T, and *R. aetherivorans* 10bc312^T were also analysed, and the type and number of their encoded gene clusters differed from those of strain T22.7.1^T.

Comparing the smBGCs of T22.7.1^T and *R. indonesiensis* CSLK01-03^T, in addition to stenothricin, there were also cinnapeptin, coelichelin, and SF2575 that were present in the former but absent in the latter; on the other hand, the smBGCs involved in the synthesis of ohmyungsamycin A/B, the rhizomide A/B/C, lymphostin/neolymphostinol B/lymphostinol/neolymphostin B, madurastatin A1/A2/E1/F/G1, and corynecin I/II/III are present in *R. indonesiensis* CSLK01-03^T, but not in strain T22.7.1^T (Supplementary Table S6). These results further illustrate the divergence in metabolic potential between strain T22.7.1^T, a new species of *R. indonesiensis*, and its developmentally most relevant strain.

Carbohydrate-active enzymes (CAZymes) are a large group of enzymes with functions related to degrading, modifying, and generating glycosidic bonds (Yin et al., 2022; Lopez-Sanchez et al., 2024). In-depth studies on CAZymes are important for understanding carbohydrate metabolism in microorganisms. Carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), auxiliary oxidoreductases (AAs), and six other protein families were annotated in the genomes of strain T22.7.1^T and its close relatives by CAZy. Strain T22.7.1^T possesses genes for synthesizing the above six classes of CAZymes, whereas polysaccharide lyase family 12/subfamily 3 is present only in strains T22.7.1^T and *R. ruber* DSM 43338^T but not in the other three reference strains (Supplementary Table S7). Reports on the functional activity of the PL 12_3 subfamily are lacking, but heparinase II/III of the PL 12 subfamily (EC 4.2.2.8) is widely used as a CAZyme for the production of clinically and therapeutically relevant bioactive heparin oligosaccharides (Balasubramaniam et al., 2018). Finally, CDA, which is of interest in this study, also belongs to one of the CAZyme and is categorized in the CE4 family, whose members are currently chitin oligosaccharide deacetylase (EC 3.1.1.-), acetylesterase (EC 3.1.1.6), acetylxylan esterase (EC 3.1.1.72), LPS deacetylase (EC 3.5.1.-), Poly-β-1,6-N-acetylglucosamine deacetylase (EC 3.5.1.-), Peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.-). acetylglucosamine deacetylase (EC 3.5.1.104), and Chitin deacetylase (EC 3.5.1.41) (Araki and Ito, 1974; Andres et al., 2014; Nakamura et al., 2017). Comparative analysis revealed that strains T22.7.1^T, *R. indonesiensis* CSLK01-03^T and *R. aetherivorans* 10bc312^T all have only one gene encoding a gene that synthesizes functional proteins of the CE 4 family, while *R. ruber* DSM 43338^T and *R. electrodiphilus* JC435^T are absent, reflecting on the one hand the functional potential of members of the genus *Rhodococcus* to produce CDA, and on the other hand suggesting that not all *Rhodococcus* strains are capable of chitin deacetylation.

Taken together, the morphological, physiological, biochemical characteristics, chemical taxonomic data, and comparative genomic analysis results of strain T22.7.1^T suggest that it is another member of the genus *Rhodococcus* that is most closely related to *R. indonesiensis* CSLK01-03^T. However, the polyphasic taxonomy results indicate that they are different, and therefore, strain T22.7.1^T is proposed as another type strain representing the *R. indonesiensis* species.

Optimization of fermentation conditions

During the initial screening of the strains, the activity of the CDA enzyme produced was tested under basal fermentation conditions. The results showed that the enzyme activity of the CDA enzyme produced by the strains under unoptimized conditions for 72 h of fermentation was 8.0 ± 2.2 U/mL. The fermentation broth was further centrifuged, and the enzyme activity in the precipitate and the supernatant was tested. The enzyme activity of the fermentation broth was taken as 100%, and the enzyme activity in the precipitate reached 90%, indicating that strain T22.7.1 produces CDA (*Ri*CDA), which is located in the bacterium and is hardly secreted outwards. Thus, all subsequent optimizations were tested with the fermentation broth to determine the enzyme activity directly after fermentation. The liquid fermentation medium and culture conditions of strain T22.7.1 were optimized by one-factor optimization and orthogonal design, aiming to increase the enzyme activity of CDA produced by the strain per unit volume, providing sufficient CDA enzyme for subsequent enzyme purification and enzymatic characterization studies. After one-way optimization, the optimal carbon source of the medium was determined to be 1.5% (w/v) sucrose, the optimal nitrogen source was 3.5% (w/v) yeast extract, the optimal inorganic salt was 0.01% (w/v) magnesium sulfate, and the optimal inducer was 0.06% (w/v) p-nitroacetanilide. Additionally, the optimized medium had an initial pH of 8, a loading volume of 30% (v/v), an inoculum volume of 4% (v/v), a fermentation temperature of 28°C, and a fermentation time of 2 days (Table 3; Supplementary Figures S3, S4). The highest enzyme activity after one-factor optimization was 287.02 U/mL, which was 35.88 times greater than that before optimization (8.0 U/mL). In contrast, the enzyme activity of *R. erythropolis* HG05, optimized by Plackett–Burman and central composite design (238.89 U/mL), was still lower than that of *R. indonesiensis* T22.7.1 (Sun et al., 2014),

TABLE 3 Optimization of fermentation conditions for CDA production of strain T22.7.1.

Fermentation conditions	Optimization condition	Enzyme activity (U/mL)
Carbon source (%, w/v)	Sucrose (1.5)	7.44 ± 0.24
Nitrogen source (%, w/v)	yeast extract (3.5)	44.44 ± 4.67
Inorganic salt (%, w/v)	Mg_2SO_4 (0.01)	10.25 ± 1.70
Inducer (%, w/v)	p-nitroacetanilide (0.06)	287.02 ± 23.19
Initial pH	8	104.84 ± 18.34
Medium volume (ml/150 mL)	50	56.81 ± 8.04
Inoculum size (%, v/v)	4	66.88 ± 1.96
Fermentation temperature (°C)	28	48.11 ± 7.94
Fermentation time (days)	2	51.44 ± 6.92

TABLE 4 Purification results of *Ri*CDA by ammonium sulfate precipitation, ultrafiltration, anion exchange chromatography, and gel filtration chromatography.

Purification steps	Volume (mL)	Protein conc. (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)	Activity yield (%)	Purification fold
Crude extract	100	6.42	242.10	37.71	100	1
Ammonium sulfate	21	9.82	936.84	95.40	81.26	2.53
Ultrafilter	17	3.27	575.91	176.12	40.44	4.67
Q Sepharose	4	0.67	383.32	572.12	6.33	15.17
Superdex 75	1	0.24	288.08	1200.33	1.19	31.83

suggesting that the T22.7.1 strain has potential application in high CDA production.

Enzyme purification, characterization and production analysis

The examination of fermentation products for the presence of CDA indicated that *Ri*CDA was intracellular. To extract the crude CDA, ultrasonic disruption of the fermented bacteria was employed, yielding a crude enzyme solution. This solution underwent a series of purification steps—75% saturation ammonium sulfate precipitation, anion exchange chromatography, and gel filtration chromatography—to achieve basic purification. From 100 mL of crude enzyme solution, 1 mL of purified CDA was obtained, representing a purification factor of 31.83-fold and a specific activity of 1200.33 U/mg, as shown in Table 4. The purification process was further assessed using SDS–PAGE, the results of which are depicted in Figure 5. The crude enzyme solution contained numerous heterogeneous proteins, which were less apparent in Line 1. After ammonium sulfate precipitation, which removed some heterogeneous proteins and concentrated the sample by 10-fold (line 2), heterogeneous proteins were still prevalent. Subsequently, a single band of pure protein was isolated through ion exchange and gel filtration chromatography. Protein electrophoresis revealed that the CDA produced by fermentation of strain T22.7.1 had an apparent molecular weight of approximately 42 kDa and was present as nearly a single component. This molecular weight is inconsistent with theoretical predictions for CE4 family proteins (consisting of 281 amino acids and a relative molecular weight of 29 kDa) annotated in the genome. Previous studies suggest that most CDAs, being glycoproteins, typically range from 40 to 80 kDa and often appear in multiple isoforms (Deising and Siegrist, 1995; Tsigos and Bouriotis, 1995; Kim et al., 2008).

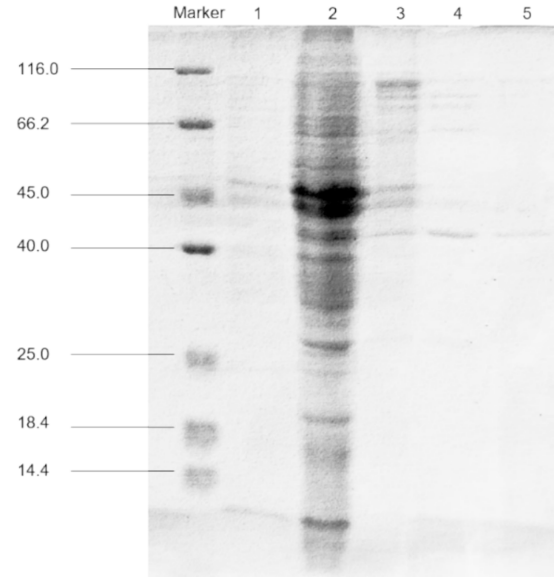

FIGURE 5
SDS-PAGE for purification analysis of CDA enzyme produced by strain T22.7.1. Marker, protein molecular weight markers; line 1, crude extract; line 2, crude CDA concentrated 10 times after ammonium sulfate precipitation; line 3, purified by Q Sepharose; line 4, purified by molecular sieve; line 5, secondary purification by molecular sieve.

Enzymatic property tests

The enzyme activity of purified *Ri*CDA was assessed under various conditions, including pH, temperature, incubation time, and reaction conditions in the presence of metal ions and chemical reagents. This investigation aimed to identify the factors that influence enzymatic deacetylation and their impact on enzyme activity. The findings revealed that the optimal pH for *Ri*CDA was 7.0, with the enzyme activity remaining above 50% of its maximum level within the range of 6.0–8.5 (Figure 6A). *Ri*CDA exhibited the highest enzyme activity at 50°C, and any deviation of 10°C above or below this temperature led to a rapid decrease in activity. Furthermore, enzyme activity was almost completely lost at temperatures of 70°C or higher due to enzyme inactivation (Figure 6B). Figures 6C,D depict the enzyme activity of *Ri*CDA under various pH buffer systems and temperatures. *Ri*CDA showed the greatest stability at pH 6.5, followed by pH 7, as evidenced by its similar performance. Even after incubation at 50°C for 8 h, the relative enzyme activity remained at approximately 50%. Notably, the enzyme activity of *Ri*CDA incubated at 50°C for 8 h did not differ significantly. Moreover, in the temperature stability test showed that at low temperature (<50°C), the enzyme activity was lost slowly decreased gradually during prolonged incubation, and the lower the temperature, the slower the at low temperatures (<50°C). After 8 h of incubation at these temperatures, the enzyme activity was maintained at approximately 80%. These data results indicate that *Ri*CDA has high exhibits remarkable stability when stored at pH 7.0 and temperatures below 30°C, which provides experience. Such insights provide valuable guidance for the subsequent efficient preservation of the *Ri*CDA enzyme.

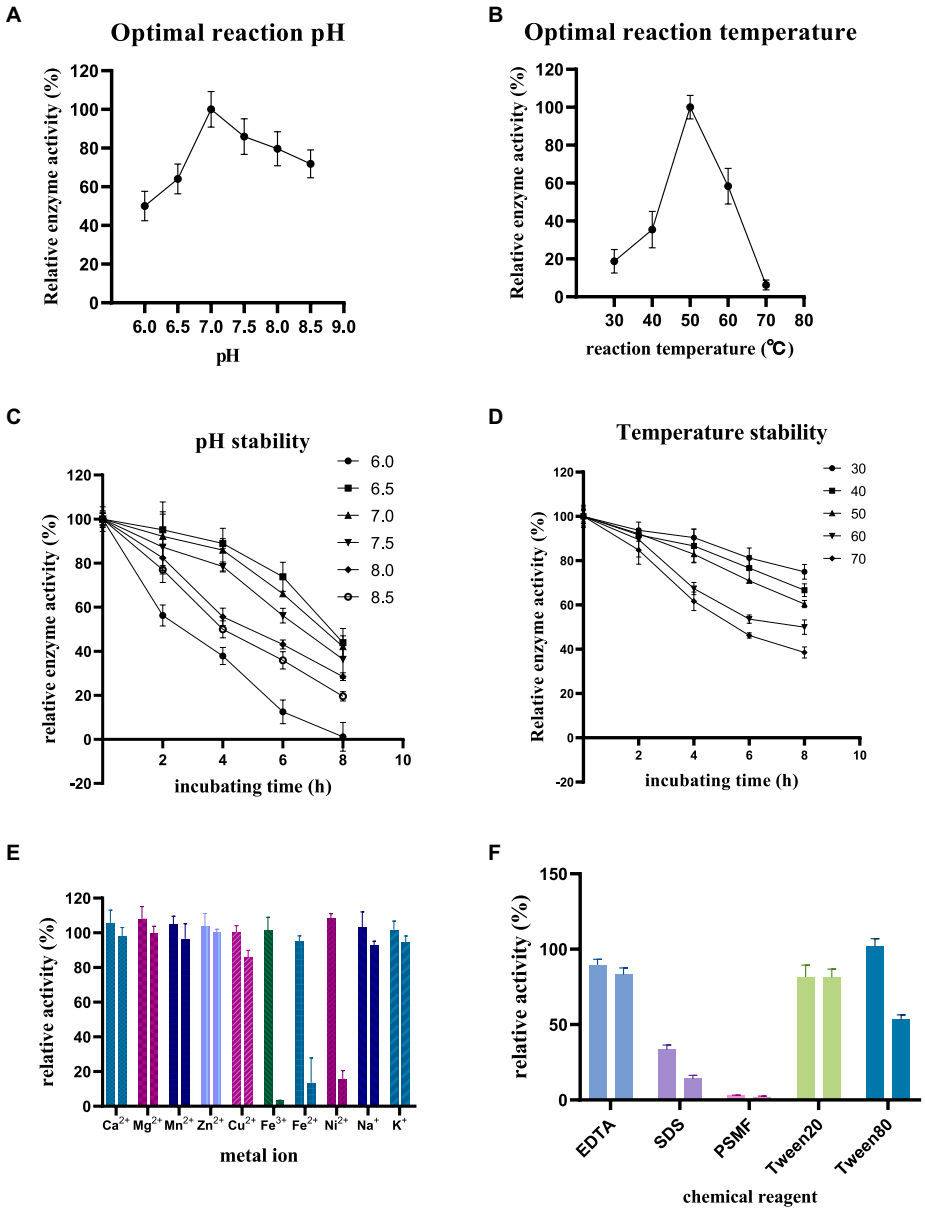

FIGURE 6
Enzymatic properties of purified *Ri*CDA **(A,B)**, Effect of pH and temperature on enzyme activity of *Ri*CDA **(C,D)**, effect of incubation at different pH and temperature for 0–8 h on enzyme activity of *Ri*CDA **(E,F)**, effect of metal ions and chemical reagents on enzyme activity of *Ri*CDA, each color represents a metal ion or chemical reagent, and the final concentration of ions in the reaction system is 1 mM on the left and 5 mM on the right. The error bar represents Mean ± SD.

Metals serve as cofactors in enzymatic catalysis and can enhance the activity of specific enzymes. To determine the metal ions essential for catalysis, the impact of various metal ions and EDTA on chitinase activity was investigated. The findings demonstrated that at low concentrations (1 mM), Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+}, and Na^{+} ions stimulated the enzyme activity of *Ri*CDA. Notably, Mg^{2+} and Ni^{2+} exhibited a more substantial promotional effect (Figure 6E), increasing the relative enzyme activity by 8.1 and 8.4%, respectively. However, compared to CDA strains from different origins (the metal ions that had a promoted metal ions up to more than doubling the relative enzyme activity), the promotion observed was less pronounced (Bai et al., 2020; Ding et al., 2021; Yang et al., 2022a). Conversely, high concentrations (5 mM) of Cu^{2+}, Fe^{3+}, Fe^{2+}, and Ni^{2+} significantly inhibited the enzyme activity of *Ri*CDA, particularly Fe^{3+}, which completely halted its activity (Figure 6E). Previous research has shown that the addition of Zn^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Ba^{2+} to the reaction system of CDA enzymes from certain bacterial strains can enhance the reaction activity. Conversely, Cu^{2+} and Ni^{2+} can substantially obstruct enzyme activity. It should be noted that the effects of these metal ions are not absolute and may yield entirely opposite effects for some CDA strains (Bai et al., 2020; Chai et al., 2020; Yang et al., 2022a). Additionally, the introduction of chemical reagents such as EDTA, SDS, and PSMF considerably hindered the activity of *Ri*CDA (Figure 6F), regardless of the concentration. The inhibition of CDA activity by EDTA has also been documented in previous studies on CDA activity from various sources. This inhibition is attributed to the ability of EDTA, as a metal ion

chelator, to chelate metal ions (e.g., Mg^{2+} and Ca^{2+} in solution), thus reducing the activity of metal-dependent proteins (Ding et al., 2021; Yin et al., 2022). SDS, an ionic surfactant, binds to proteins, leading to denaturation and precipitation. Conversely, the inhibition of *Ri*CDA activity by phenylmethylsulfonyl fluoride (PMSF) may be ascribed to the specific binding of PMSF, a serine protease inhibitor, to serine residues at the enzyme's active site, resulting in enzymatic inhibition (Valenzuela et al., 2023; Rani et al., 2024). Furthermore, Tween 20 mildly inhibited *Ri*CDA activity, whereas at a low concentration (1 mM), Tween 80 promoted *Ri*CDA activity but significantly suppressed enzyme activity at a high concentration (5 mM) (Figure 6F). This can be attributed to the fact that Tween 80 acts as a surfactant, stabilizing proteins through interfacial competition at low concentrations. Conversely, at high concentrations, Tween 80 reduces the accessibility of the enzyme's active site, leading to decreased enzyme-substrate affinity (Fahmy and El-Deeb, 2023).

The substrate preference of *Ri*CDA for various substrates, including chitin, chitosan, and COS, was determined using HPLC to analyse the change in acetic acid content in the enzymatic deacetylation products. Among these substrates, NAG, CTO, and CSO are all water soluble. The results indicated that *Ri*CDA exhibited the highest enzyme activity towards CTO, followed by NAG, and the lowest activity towards CSO, suggesting a strong affinity of the CDA enzyme for acetylated CTO and NAG. Furthermore, the different durations of the enzymatic reaction of CTO demonstrated that the enzyme activity of *Ri*CDA decreased as the reaction time increased. However, even so, compared to that of the insoluble substrates chitin and chitosan, the enzyme activity of *Ri*CDA was highest for CTO when the reaction time was the same (3 h) (Table 5). Additionally, the deacetylation activity of *Ri*CDA was greater for chitin than for chitosan, possibly due to the higher acetylation level of chitin. In conclusion, *Ri*CDA exhibited deacetylation activity for all the mentioned substrates but displayed higher activity for substrates with a higher degree of N-acetylation and much higher activity for CTO than for CSO.

Product characterization

The deacetylation products of CTO and NAG were analysed using TLC. When the acetyl group in oligosaccharides or monosaccharides is converted to an amino group, the polarity is increased, resulting in smaller retention factor values (Rf values) and shorter distances travelled by more polar substances (Rani et al., 2024). The results of the enzymatic products of *Ri*CDA are shown in Figure 7. The standard sample of CTO, which also includes NAG, was enzymatically deacetylated, resulting in a position lower than that of the standard CTO samples on TLC silica gel plates. This indicates that both the NAG and CTO of DP2-5 can be deacetylated by *Ri*CDA. In Figure 7A, spots very close to the spotting origin can be clearly observed in the 1 h, 3 h, and 6 h *Ri*CDA-treated groups. These spots correspond to completely deacetylated CSO, with the smallest Rf value due to the maximum polarity of CSO compared to that of CTO. Similar results were observed for *Ri*CDA-deacetylated NAG, chitobiose, and chitotriose (ex1, ex2, ex3), as shown in Figure 7B. Additionally, previous studies have shown that different sources of chitin deacetylases exhibit different patterns of enzymatic action on COS substrates (Naqvi et al., 2016; Grifoll-Romero et al., 2018; Bai et al., 2020; Bonin et al., 2020).

Scanning electron microscopy (SEM) was used to characterize the α-chitin-, chitosan-, and *Ri*CDA-treated chitin (*Ri*CDA-chitin). The SEM results depicted in Figure 8 show that the untreated α-chitin exhibited a large, dense, layered structure in a lamellar arrangement, with a surface composed of closely packed crystalline microfibrillar structures that were dense, rough, and textured. However, in the case of *Ri*CDA-treated chitin, the original dense lamellar structure was profoundly damaged, resulting in the appearance of numerous irregular pits. The surface of *Ri*CDA-chitin exhibited holes and dense cracks, whereas its fibres became scattered and fuzzy, and the interface with the separated lamellae was less distinct. The particle state of *Ri*CDA-chitin resembled that of chitosan, which differed significantly from that of α-chitin (Yang et al., 2022a,b). As *Ri*CDA deacetylation results in a decrease in the acetyl group content on the glycan chains of chitosan, it leads to the disruption or weakening of the hydrogen bonding that maintains the intramolecular and intermolecular structural stability of the glycan chains, which results in the change of the microstructure of chitosan (Yang et al., 2023).

To identify the characteristic groups of *Ri*CDA-chitin, Fourier transform infrared spectroscopy (FT-IR) was conducted, and the results were compared with those of α-chitin and chitosan. The results, as presented in Figure 9, displayed a broader absorption peak at approximately 3,400 cm^{-1}, resulting from the overlapping of -OH stretching vibrational peaks forming hydrogen bonding and -NH stretching vibrational peaks. Other significant peaks included the -CH stretching vibration at 2,928 cm^{-1}, the amide I band at 1635 cm^{-1}, and the amide II band at 1,558 cm^{-1}. Additionally, the NH stretching vibration absorption peak overlapped and broadened multiple absorption peaks, including the -CH stretching vibration absorption

TABLE 5 Enzyme activity of *Ri*CDA for different substrates. -, presents no test.

Substrate	Enzyme activity(U/mg)		
	Enzymatic reaction time (h)		
	1	3	6
10 mg/mL NAG	52.42 ± 4.48	–	–
20 mg/mL CTO	217.58 ± 12.33	91.12 ± 8.02	75.08 ± 10.23
20 mg/mL CSO	25.42 ± 2.31	–	–
50 mg/mL chitosan	–	2.92 ± 0.02	–
50 mg/mL chitin	–	27.75 ± 5.62	–

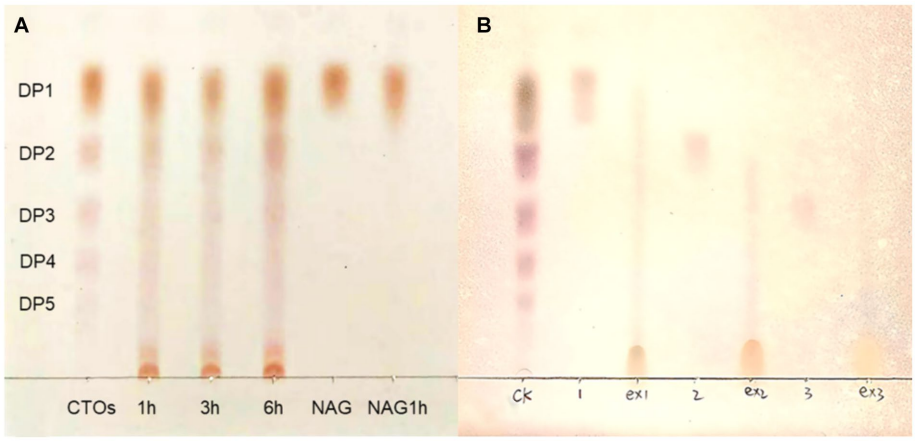

FIGURE 7
TLC results of chitin oligosaccharides deacetylated by *Ri*CDA. **(A)** CTOs was treated by *Ri*CDA for different times. **(B)** Comparison of oligosaccharides with 1–3 degree of polymerization before and after *Ri*CDA treatment.

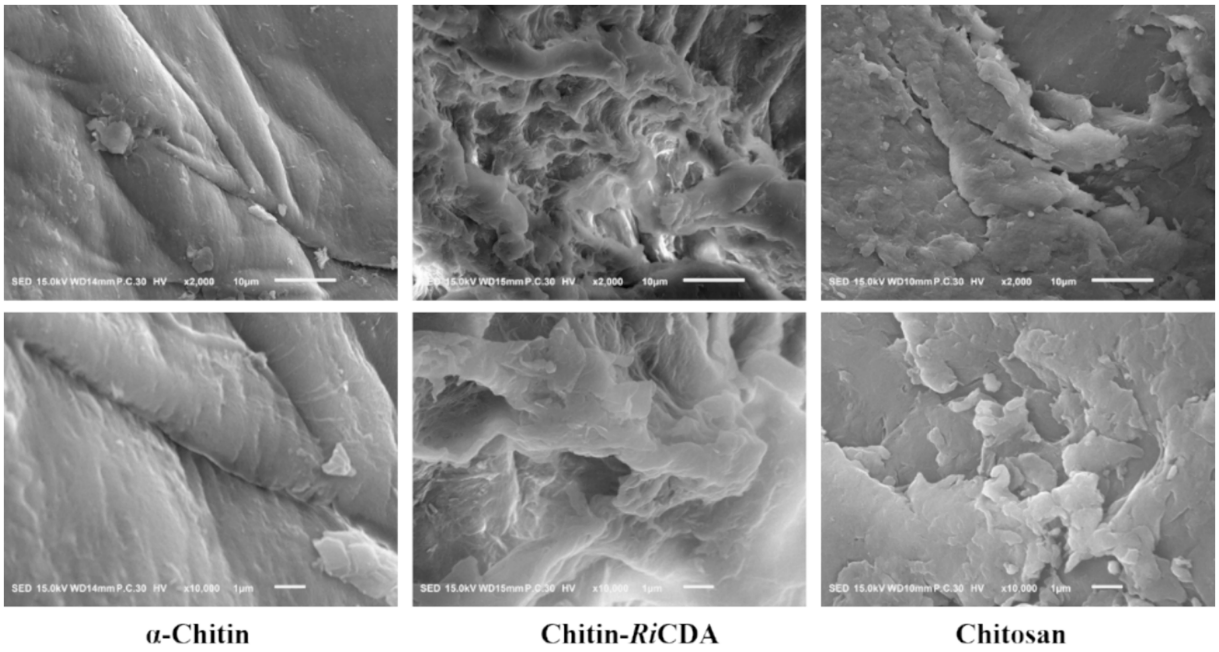

FIGURE 8
SEM images of untreated chitin, chitin treated by *Ri*CDA and chitosan with magnificent ×2,000 times (upper layer) and ×10,000 times (under layer).

peak at 2,928 cm^{-1}, amide I spectral band at 1,635 cm^{-1}, -NH deformation vibration absorption peak of amide II at 1,558 cm^{-1}, 1,381 cm^{-1} for the -CH2 wobble absorption peak, amide III spectral band at 1,316 cm^{-1}, asymmetric oxygen bridge telescopic vibrational absorption peak at 1,158 cm^{-1}, and C-O telescopic vibrational absorption peaks at 1,073 and 1,027 cm^{-1} (Chai et al., 2020; Yang et al., 2022b, 2023). Notably, the -CH stretching peak at 2,928 cm^{-1} exhibited a shift to lower frequencies in correlation with the higher crystallinity of the chitin (Yang et al., 2022a). It is evident from the figure that the *Ri*CDA treatment resulted in relatively low crystallinity of the chitin. The DD was determined by the weakening of NH (3,269 cm^{-1}), the C=O linkage of amide I (1,633 cm^{-1}), the N-H linkage of amide II (1551–1,546 cm^{-1}), and the band of amide III (1,378 cm^{-1}) (Sixto-Berrocal et al., 2023). For analytical purposes, the amide I and III bands (1,655, 1,560, and 1,320 cm^{-1}) were selected to calculate the deacetylation of chitin, chitosan, and *Ri*CDA-chitin due to the difficulty in determining the amide II band at high DDs. The results revealed that the DD of α-chitin was 36.43%, whereas that of *Ri*CDA-chitin was 83.70%, surpassing that of commercial chitosan (78.73%). Most of the identified CDAs have been reported to exhibit high activity against low molecular weight oligomers, whereas they are largely inactive against natural insoluble chitin (Kang et al., 2014; Naqvi et al., 2016). In this study, *Ri*CDA was found to be an effective chitinolytic agent to catalyze the deacetylation of chitin for the preparation of chitosan and to achieve value-added utilization of chitin bioresources.

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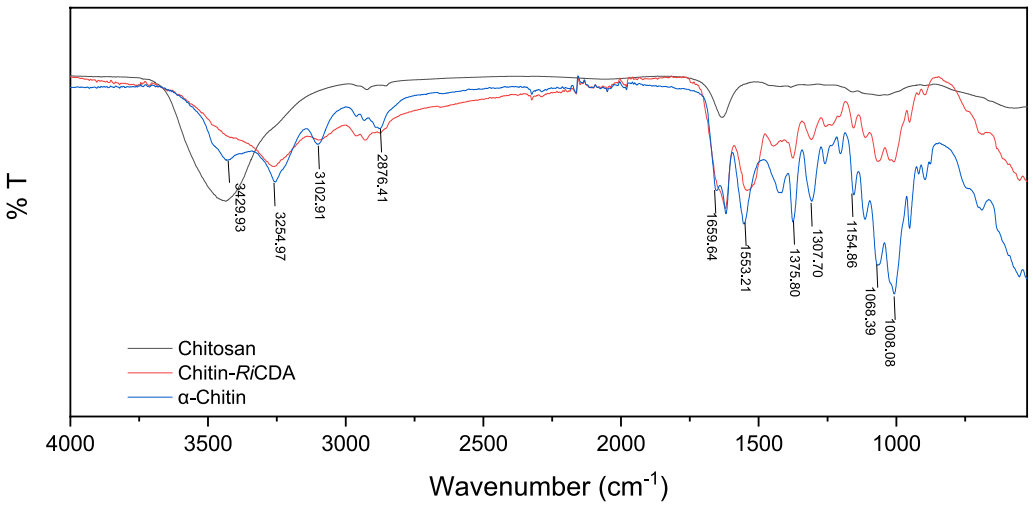

FIGURE 9
FT-IR spectra of chitin, chitosan, and *Ri*CDA treated chitin. Chitosan (black), *Ri*CDA treated chitin (red) and chitin (blue).

Conclusion

Conclusions on the species identification of the new isolate

Based on the genomic, phylogenetic, and phylogenomic data, it can be inferred that strain T22.7.1^T is not only a member of the genus Rhodococcus but also a member of the *R. ruber* lineage. Through the use of phenotypic traits and the examination of dDDH and ANI values, it was possible to distinguish strain T22.7.1^T from its close relatives *R. aetherivorans* DSM 44752^T, *R. electrodiphilus* LMG 29881^T, and *R. ruber* DSM 43338^T. However, differentiating it from *R. indonesiensis* CSLK01-03^T in terms of species status has proven challenging. Considering the taxonomic characteristics described earlier, the new isolate and *R. indonesiensis* CSLK01-03^T can be regarded as distinct type strains representing *R. indonesiensis*. Importantly, these associations are supported by ANI and dDDH similarities that exceed the thresholds used to classify closely related strains as the same species. Therefore, we propose that strain T22.7.1^T be designated as a post heterotypic synonym of *R. indonesiensis* (Kusuma et al., n.d.).

Emended description of *Rhodococcus indonesiensis* (approved lists 2014)

Description is based on data from this and previous studies (Kusuma et al., n.d.).

Aerobic, Gram-stain-positive, non-motile actinomycetes, forming short rods (0.8–1.5 or 1.8–3.2 μm) and spherical elements (0.9–1.1 or 1.2–1.6 μm). The cell surface was smooth and showed cap-like projections at the ends, and the colony shifted from yellowish white to orange-red on ISP2 medium. They grew well on ISP series medium ISP1, ISP2, ISP3 and ISP6, were able to grow at temperatures ranging from 10 to 45°C, with an optimal growth temperature range of 28–35°C, were able to grow at pH ranging from 5.0–9.0, with an optimal pH around 7.0–8.0, were able to tolerate NaCl concentrations of 0–12% (w/v), and were able to grow at an optimal NaCl concentration of 1% (w/v). Catalase positive, oxidase negative, reduces nitrate, produces H_2S, hydrolyzes picrin, allantoin, arbutin, gelatin, arginine and urea, produces lysine decarboxylase, does not produce phenylalanine deaminase. Degrades adenine, hypoxanthine, and uric acid but not casein, elastin, guanine, keratin, starch, casein tributyrate, xanthine, Tweens 20, 60, and 80. inositol, sodium gluconate, D-mannitol, D-sorbitol, maltose, NAG, cellulosic disaccharides, D-galactose, D-glucose, D-fructose, D-cotton seed sugars, glycerol, D-ribose, D-arabinose, trehalose, D-mannose, sucrose, L-rhamnose can be used as the sole carbon source, but arbutin, lecithin, cellulose, and sodium butyrate cannot. The characteristic diamino acid of the cell wall is meso-DAP. whole-cell hydrolysate sugars contain galactose, arabinose, or glucose. The major cellular fatty acids (>10%) were $C_{16:1}$ ω6c/ω7c, $C_{16:0}$, $C_{18:0}$ 10-methyl-TBSA, the major methylnaphthoquinone (>10%) was MK-8 (H2) and the diagnostic polar lipids were diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, phosphatidylglycerol. Produces mycolic acid. The strain has a genome size of about 5.5 Mbp and a DNA G + C content of about 70.15 mol%.

Strain T22.7.1^T (=MCCC 1K08698^T = JCM36625^T) was isolated from the rhizosphere of *Acanthus ebracteatus* in Beihai City, Guangxi Zhuang Autonomous Region, China. The GenBank accession number of the strain is JASKMB000000000.

Fermentation optimization of strain T22.7.1, enzymatic properties and product characterization of *Ri*CDA

After optimizing the fermentation medium composition and culture conditions of strain T22.7.1, the enzyme activity per unit volume of the cultured strain reached 287.02 U/mL after 48 h. Additionally, the yield of CDA increased by 34.88 times compared to the non-optimized condition. This is solely the result of single-factor optimization. It is believed that further optimization of the fermentation process of strain T22.7.1 can significantly increase its CDA yield. The enzyme activity of CDA produced by the fermentation of T22.7.1 reached the highest reported level among strains of the same genus (Sun et al., 2014; Ma et al., 2020). *Ri*CDA

was extracted and purified using a series of procedures, including cell wall disruption, ammonium sulphate precipitation, anion exchange chromatography, and gel filtration chromatography. The actual molecular weight of *Ri*CDA was approximately 42 kDa, which differed from the theoretical prediction of 29 kDa for CE4 family proteins noted in the genome. Previous studies have shown that *Ri*CDA is a glycoprotein with a protein subunit structure that differs from theoretical CDA molecules.

The purified *Ri*CDA exhibits excellent adaptability and stability to pH and temperature, and is capable of deacetylation activity within the pH range of 6.0–8.5 and temperature range of 30–70°C. Furthermore, it remains active even after continuous storage for 8 h within this range. *Ri*CDA exhibits the highest enzyme activity at pH 7.0 and 50°C. It can maintain approximately 70% enzyme activity for 8 h under these conditions, demonstrating its potential in the industrial deacetylation of chitin or chitin oligosaccharides for the preparation of chitosan and chitosan oligosaccharides. On the other hand, at low concentrations (1 mM), Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and other metal ions have a certain effect on the activity of *Ri*CDA, while high concentrations (5 mM) of Cu^{2+}, Fe^{3+}, and Fe^{2+} ions will inhibit its activity. Additionally, regardless of concentration, EDTA, SDS, PMSF, and Tween 20 inhibited the enzyme activity of *Ri*CDA, while Tween 80 helped to increase enzyme activity at low concentration and inhibited enzyme activity at high concentration. Regarding substrate adaptability, *Ri*CDA has a broad spectrum and can effectively deacetylate various substrates, such as chitin, chitosan, CTO, CSO, and NAG. Among these, CTO are the most suitable substrate, followed by NAG. *Ri*CDA also demonstrated deacetylation ability for chitin oligosaccharides with a polymerization degree of 1–5, further highlighting its potential applications.

Through the deacetylation of *Ri*CDA, natural chitin can be transformed into chitosan by removing the acetyl group on the sugar chain. This process reduces the structural stability of chitin and enhances its solubility, making it more conducive to degradation. In conclusion, strain T22.7.1 is a newly discovered strain with a relatively strong chitin deacetylation function. It has a controllable fermentation process to optimize the production of CDA and a considerable yield. The *Ri*CDA produced exhibits superior pH and temperature adaptability and stability, has a wide range of substrates, and is easy to purify and prepare. These advantages make it an important candidate for the bioconversion of chitin into high value-added products using a bioenzymatic method. Furthermore, it provides a key element for further research on chitin biorefining.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

JX: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. DoY: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. JO: Writing – original draft, Visualization, Validation, Methodology, Data curation, Conceptualization. BL: Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. SL: Writing – review & editing, Validation, Supervision, Project administration, Methodology. DeY: Writing – review & editing, Validation, Supervision, Methodology. HZ: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. NS: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1427143/full#supplementary-material

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Comparison of microbiota structure in reproductive tract of Yanbian cattle and Yanhuang cattle

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Microbiota in the reproductive tract of cattle play a vital role in maintaining normal reproduction. However, the information on microbiota in different parts of reproductive tracts with different genetic background is few. The aim of the present study was to describe and compare the microbiota in vagina, cervix and uterus of Yanbian cattle and Yanhuang cattle. The results showed that microbial diversity increases from the vagina to the uterus. The top three bacterial phyla in bovine reproductive tract were *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, accounting for more than 85%. From the vagina to the uterus, the relative abundance of *Proteobacteria* gradually decreased, while that of *Firmicutes* gradually increased. Phylum-level *Firmicutes* and genus-level *UCG_010* were significantly enriched in the uterus of Yanbian cattle and Yanhuang cattle. Comparing the same parts of the two breeds, it was found that there was no significant difference in alpha diversity, but significant differences in beta diversity. In addition, microbiota with significant differences in the relative abundance of the reproductive tract were found. These findings lay a foundation for a comprehensive understanding of the structure of the genital tract microbiota of cows and its regulatory mechanisms.

KEYWORDS

Yanbian cattle, Yanhuang cattle, microbiota, reproductive tract, 16S rRNA gene

Introduction

The genital tract microbiota plays an important role in cattle reproductive health. The reproductive tract microbiota can inhibit the invasion and proliferation of pathogens by forming biofilms (Tang et al., 2008; Tachedjian et al., 2017). Certain lactobacilli can protect fetal development during pregnancy and promote healthy delivery (Romero et al., 2014). Recent research shows that reproductive tract microbiota can transmit chemical signals between species by producing pheromones (Srinivasan et al., 2021). The dysbiosis leads to changes in the microbiota, including a decrease in the abundance of lactobacilli and an increase in the population of facultative anaerobes, leading to a predisposition of the host to a variety of diseases (Valenti et al., 2018; Saraf et al., 2021). Exploring the reproductive tract microbiota structure of

healthy cattle will provide a solid theoretical basis for studying the occurrence of reproductive diseases and reproductive obstacles.

However, the microbes in the reproductive tract are not fixed. Bacteria can enter the vagina from the outside, including skin and feces, and transmitted to sites such as the cervix and uterus (Sheldon and Dobson, 2004). Bacteria can also enter the reproductive tract through the bloodstream route (Jeon et al., 2017). The vagina is still considered the main source of microbiota in the uterus, cervix and other parts of the body (Galvão et al., 2019). Pathogenic bacteria that cause uterine infections such as *Prevotella*, *Fusobacterium necrophorum*, *Escherichia coli*, *Arcanobacterium pyogenes*, etc. are often proven to be associated with the vagina (Deng et al., 2019). Therefore, the microbiota composition of the vagina, cervix, and uterus is expected to be closely related. Although previous studies have separately reported the structure of vaginal microbiota and uterine microbiota in cows (Vitale et al., 2021), and there is a lack of systematic exploration of the complete reproductive tract microbiota.

For studying the composition of microbiota in various parts of the cow's reproductive tract, in addition to studying the correlation between the microbiota in various parts, its changes under breed factors should also be considered. Studies on Gyr (Giannattasio-Ferraz et al., 2019) and Nellore (Laguardia-Nascimento et al., 2015) cattle found significant differences in vaginal microbiota. However, previous studies on the impact of genetic factors on reproductive tract microbiota may be more affected by sampling region, season and nutritional factors, and there is a lack of research on the cervix and uterus. For this reason, it is necessary to eliminate interfering factors such as region, feeding management and feed differences that affect the reproductive tract microbiota as much as possible, and systematically study the impact of breed factors on the entire reproductive tract microbiota.

Yanbian cattle, one of the five major local fine-bred cattle in China, originated from 1850 to 1870 and was formed by cross-breeding Korean cattle and Mongolian cattle (Shen et al., 2020). Yanhuang cattle are made from Limousin cattle as the male parent and Yanbian cattle as the female parent, through cross-breeding, cross-fixation and group selection, this breed contains 75% of Yanbian cattle genes and 25% of Limousin cattle genes. Compared with Yanbian cattle, the growth and development of Yanhuang cattle at various stages has been significantly improved than that of Yanbian cattle. Yanhuang cattle have obvious advantages in slaughter performance and feed conversion ratio, and the digestibility of dietary nutrients is also higher than that of Yanbian cattle (Ji et al., 2014).

The aim of the study is to investigate the commonality and uniqueness of microorganisms in different parts of the reproductive tract of Yanbian cattle and Yanhuang cattle, as well as the influence of breed factors on the composition of microorganisms in the bovine reproductive tract, which laid a foundation for a comprehensive understanding of the microecological composition and regulation of bovine genital tract.

Methods

Animals and samples

This study selected Yanbian cattle and Yanhuang cattle from Benfu Ranch in Yanbian Korean Autonomous Prefecture, China. Multiparous cattle with healthy body condition and aged 3–5 years were selected. Samples were collected from November and December 2022. In order to avoid cows in different physiological cycles affecting the structure of the reproductive tract microbiota, we use estrus identification technology to select cows that were naturally in estrus (external observation combined with vaginal examination). Cotton swabs was used to collect samples on the day of estrus, and samples from the reproductive tract including the vagina, cervix and uterus were collected. A total of 98 samples from 21 Yanbian cattle (vagina: $n = 14$; cervix: $n = 17$; uterus: $n = 17$) and 19 Yanhuang cattle (vagina: $n = 17$; cervix: $n = 17$; uterus: $n = 16$) were collected for subsequent sequencing analysis. Samples were collected on sterile cotton swabs, placed in cryovials, group the samples (A.V: Yanbian cattle vagina; A.C: Yanbian cattle cervix; A.U: Yanbian cattle uterus; B.V: Yanhuang cattle vagina; B.C: Yanhuang cattle cervix; B.U: Yanhuang cattle uterus), and stored in liquid nitrogen at −196°C until used for microbiome analysis.

DNA extraction and sequencing

Genomic DNA was extracted by CTAB method, and DNA purity and concentration were detected using 1% agarose gel electrophoresis. Take an appropriate amount of sample DNA in a centrifuge tube and use sterile water to dilute the sample to 1 ng/μL. PCR amplification of the bacterial 16S rRNA gene V3–V4 region was performed using the forward primer 341F (5′-CCTAYGGGRBGCASCAG -3′) and the reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Table 1). Add 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM primer and 10 ng genomic DNA template to the PCR mixture, perform the first denaturation at 98°C for 1 min, and then denature at 98°C (10 s) for 50°C (30 s) and 72°C (30 s) for 30 cycles, and it was maintained at 72°C for 5 min to obtain the PCR product. The expected amplified product fragment was 470 bp. PCR products are detected by electrophoresis using 2% concentration agarose gel; qualified PCR products are purified with magnetic beads and quantified using enzyme labeling. Equal amounts of samples are mixed according to the concentration of the PCR product. After mixing thoroughly, use 2% agarose gel electrophoresis to detect the PCR product, and use a universal DNA purification and recovery kit (TianGen) to recover the product of the target band. NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs) was used for library construction. The constructed library was quantified by Qubit and Q-PCR. After the library was qualified, NovaSeq 6000 (518 cycles) was used for PE 250 On-machine sequencing. All sequences used in this study are publicly available at the NCBI Sequence Read Archive under accession ID PRJNA1129596.

TABLE 1 Primers sequences for PCR.

Region	Primer name	Primer sequence (5′–3′)	Product (bp)
Bacteria 16S	V3 + V4	341F	CCTAYGGGRBGCASCAG
		806R	GGACTACNNGGGTATCTAAT

Sequence analysis

Illumina NovaSeq sequencing platform was used for double-terminal sequencing of the library. Briefly, according to the Barcode sequence and PCR amplification primer sequence, each sample data is split from the offline data. Double-end data splicing uses FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/) to truncate the Barcode and primer sequences to splice the reads of each sample, and the resulting spliced sequence is: Raw Tags data (Raw Tags). Use fastp software (Version 0.23.1) to perform strict filtering on the spliced Raw Tags to obtain high-quality Tags data (Clean Tags). The Tags obtained after the above processing need to be processed to remove chimera sequences. The Tags sequences are compared with the species annotation database (Silva database https://www.arb-silva.de/ for16S) to perform comparison and detection of chimeric sequences, and finally remove the chimeric sequences to obtain the final effective data (Effective Tags). For the obtained EffectiveTags, the DADA2 module or deblur in the QIIME2 (VersionQIIME2-202006) software was used for denoising (DADA2) to obtain the final ASVs (Amplicon Sequence Variants) and feature tables. Classify-sklearn algorithm of QIIME2 is adopted (Bokulich et al., 2018; Bolyen et al., 2019) annotated species for each ASV using a pre-trained Naive Bayes classifier.

Bioinformatic analysis

Sequence data analysis was mainly performed using the QIIME2 and R packages. According to the results of ASVs annotation and the characteristic table of each sample, the top 10 dominant species were screened from the species abundance table at phylum and genus level, and the relative species abundance histogram was generated. A Venn diagram was generated to visualize the shared and unique OTUs among samples or groups using the R (Version 3.5.3) package "Venn Diagram." Use QIIME2 software to calculate α diversity indices such as Chao1 index and Shannon index. β-diversity analysis was performed using the Bray–Curtis index to investigate structural changes in microbial communities in the sample and visualized by generating PCoA distribution maps. LDA effect size (LDA score >4) was performed to detect differentially abundant taxa across groups using the default parameters. In R (Version 3.5.3), a *t*-test was used to compare the abundance of taxa at the phyla and genus level within the group.

Results

Comparison of vaginal, cervical and uterine microbiota

Through the species relative abundance column chart, we can visually see the species and their proportions with higher relative abundance in each group at the phylum and genus level. As shown in Figure 1A, the top three dominant bacterial phyla in the reproductive tract of Yanbian cattle are *Proteobacteria* (uterus: 38.63%, cervix: 47.65%, vagina 59.97%), *Firmicutes* (uterus: 41.19%, cervix: 34.56%, vagina: 21.27%), *Bacteroidota* (uterus: 12.90%, cervix: 10.11%, vagina: 9.97%). The proportion of the top three dominant bacterial phyla in various reproductive tract parts

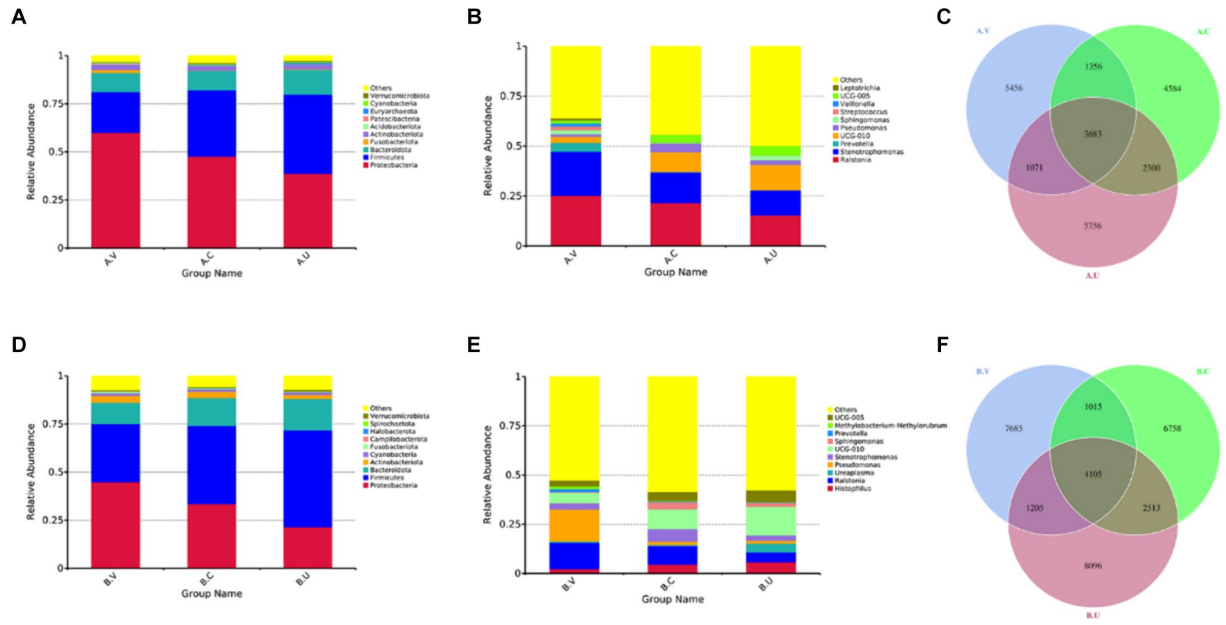

FIGURE 1
Composition and proportion of vaginal, uterine and cervical microbiota. **(A,B,D,E)** are the species annotations and abundance information at the phylum and genus level of the vagina, cervix, and uterus of Yanbian cattle and Yanhuang cattle. The top 10 species with the highest abundance were selected to generate a column accumulation chart of species relative abundance, the abscissa is the group name; the ordinate represents the relative abundance; others represents the sum of the relative abundance of all other phyla except these 10 phyla in the figure. Each circle in the figures **C,F** represents a group. The number in the overlapping part of the circle and the circle represents the number of OTUs shared between the groups. The number in the non-overlapping part represents the number of unique OTUs of the group.

of Yanbian cattle is as high as 91.35–92.72%. The top three bacterial phyla in the reproductive tract of Yanhuang cattle are *Proteobacteria* (uterus: 21.37%, cervix: 33.60%, vagina: 30.30%), *Firmicutes* (uterus: 50.48%, cervix: 40.50%, vagina: 30.30%), *Bacteroidota* (uterus: 16.25%, cervix: 14.58%, vagina: 11.12%) (Figure 1D). The proportion of the top three dominant bacterial phyla in various reproductive tract parts of Yanhuang cattle is as high as 86.18–88.68%. The results showed that the top three dominant phyla of the two breeds of cattle were *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, and these three phyla showed a consistent trend in the reproductive tract: the relative abundance of *Proteobacteria* showed a gradient decreasing trend from the vagina to the uterus, and the relative abundance of *Firmicutes* showed a gradient increasing trend from the vagina to the uterus, there was no obvious change in the *Bacteroidetes* phylum. From the genus level, *Ralstonia* occupies a high abundance in the reproductive tract of Yanbian cattle and Yanhuang cattle, and the relative abundance gradually decreases from the vagina to the uterus. As another dominant genus, *UCG-010* has a relative abundance that gradually increases from the vagina to the uterus of Yanbian cattle and Yanhuang cattle. The *Histophilus* only occupies a greater advantage in the reproductive tract of Yanhuang cattle, and the relative abundance of *Histophilus* showed an increasing trend from the vagina to the uterus of Yanhuang cattle (Figures 1B,E).

As shown in Figures 1C,F, the number of OTUs that coexisted in the vagina, cervix and uterus of Yanbian cattle reached 3,683, 5,039 OTUs coexisted in the vagina and cervix, 5,983 OTUs coexisted in the cervix and uterus, and 4,754 OTUs coexisted in the vagina and uterus. The number of OTUs that coexisted in the vagina, cervix and uterus of Yanhuang cattle reached 4,105, 5,120 OTUs coexisted in the vagina and cervix, 6,618 OTUs coexisted in the cervix and uterus, and 5,310 OTUs coexisted in the vagina and uterus. In addition, there are still relatively high amounts of OTU in the vagina, cervix, and uterus of the two breeds of cattle, which are unique to each part.

By comparing the differences in Shannon index and Chao 1 index of the vagina, cervix and uterus of Yanbian cattle and Yanhuang cattle (Figures 2A,B,E,F), it was found that the alpha diversity changes of the reproductive tract microbiota of Yanbian cattle and Yanhuang cattle showed consistent changes. There was a significant difference in the alpha diversity of the vagina and uterus between the two breeds ($p < 0.05$). However, there was no significant difference in alpha diversity between vagina-cervix and cervix-uterus ($p > 0.05$). Based on the PCoA distribution of vaginal, cervical and uterine samples from the two breeds of cattle, it was found that the distribution of vaginal samples was significantly different from the distribution of uterine and cervical samples, while the distribution of cervical and uterine samples was more similar and the community structure was more similar (Figures 2C,G). In order to further determine the significance of distribution differences between sample groups, using the Wilcox rank sum test method based on Bray–Curtis, there were significant differences in vaginal-cervical and vaginal-uterine beta diversity ($p < 0.05$), and there was no significant difference in cervical-uterine beta diversity ($p > 0.05$) (Figures 2D,H).

In order to find out the species with significant differences in different genital tract parts between Yanbian cattle and Yanhuang cattle, statistics of significantly enriched bacterial groups in each group were carried out through LEfSe analysis. As shown in Figure 3. *o_Veillonellales_Selenomonadales* was significantly enriched in the vagina of Yanbian cattle, and

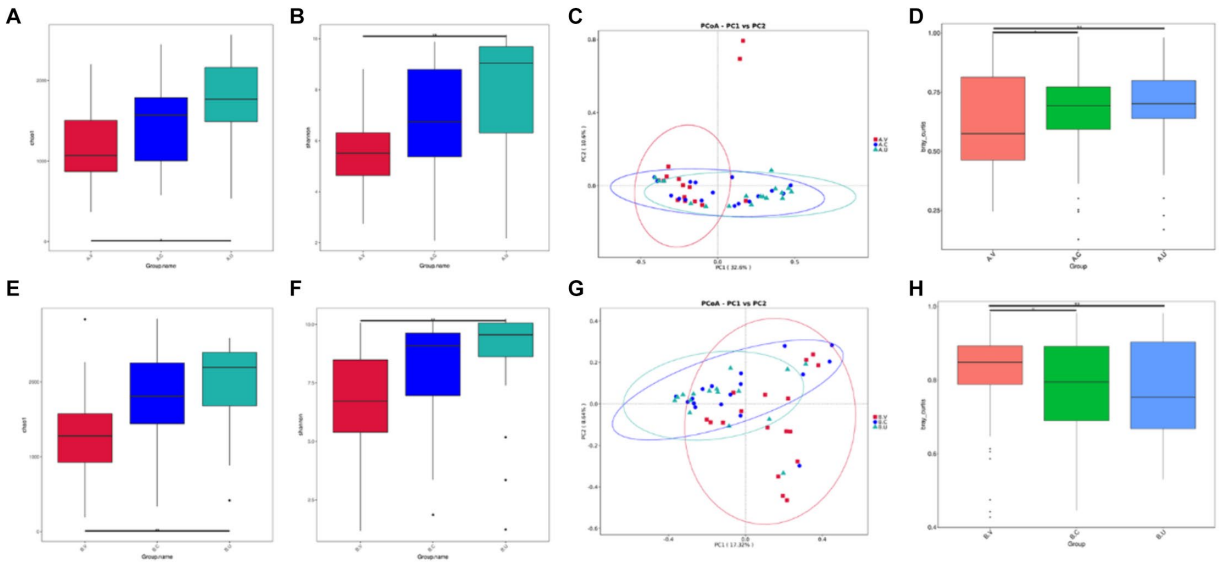

FIGURE 2
Comparison of the alpha and beta diversity of the vaginal, cervical and intrauterine microbiota of Yanbian cattle and Yanhuang cattle. Alpha diversity, including Chao 1 and Shannon index **(A,B,E,F)** of the two groups of samples, **C,G** based on the beta diversity of the Bray–Curtis metric of Yanbian cattle and Yanhuang cattle. The abscissa represents one principal component, the ordinate represents another principal component, and the percentage represents the contribution of the principal component to the sample difference; each point in the figure represents a sample, and samples in the same group are represented by the same color. **(D,H)** The Wilcox rank sum test was used to analyze the Bray–Curtis differences among each group. *Means significant difference ($p < 0.05$), **means extremely significant difference ($p < 0.01$).

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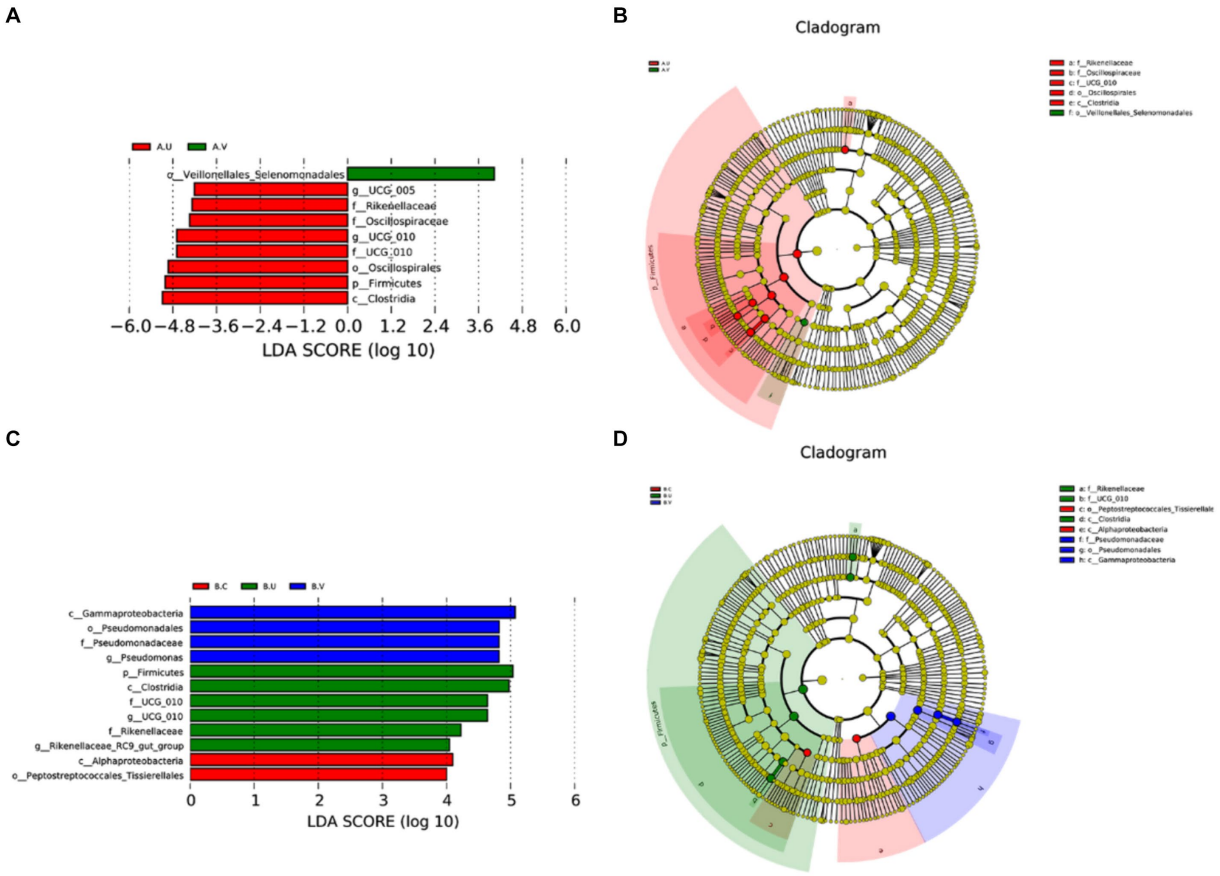

FIGURE 3
Comparison of the microbiota in the vagina, cervix and uterus of Yanbian cattle and Yanhuang cattle. The LDA value distribution histogram shows species whose LDA score is greater than the set value of 4, that is, species that are significantly enriched in each group. The length of the bar graph represents the effect size (LDA score) of differential species **(A,C)**. In a cladogram, the circles radiating from the inside to the outside represent the classification levels from phylum to genus (or species). Each small circle at a different classification level represents a classification at that level, and the diameter of the small circle is proportional to the relative abundance **(B,D)**.

c_Gammaproteobacteria, *o_Pseudomonadales*, and *f_Pseudomonadaceae* were significantly enriched in the vagina of Yanhuang cattle. In the uterus of Yanbian cattle and Yanhuang cattle, *f_Rikenellaceae*, *g_UCG_010*, *f_UCG_010*, *p_Firmicutes*, and *c_Clostridia* were significantly enriched. *g_UCG-005*, *f_Oscillospiraceae*, and *o_Oscillospirales* were only enriched in the uterus of Yanbian cattle. *g_Rikenellaceae_RC9_gut_group* was only significantly enriched in the uterus of Yanhuang cattle. Thus, phylum-level *Firmicutes* and genus-level *UCG_010* were significantly enriched in the uterus of two breeds of cattle, *UCG-005* was significantly enriched only in the uterus of Yanbian cattle, and *Rikenellaceae_RC9_gut_group* was significantly enriched only in the uterus of Yanhuang cattle (Figures 3A,C).

Comparison of microbiota in the same reproductive tract between Yanbian cattle and Yanhuang cattle

In order to study the main composition and proportion of microbiota in the same reproductive tract of Yanbian cattle and Yanhuang cattle, the top 10 species with the highest abundance were selected to generate a column accumulation chart of species relative abundance. In order to visually observe the distribution of dominant species among groups. The results showed that at the phylum level, the top three bacterial phyla were *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Among them, the relative abundance of *Proteobacteria* in the vagina, cervix and uterus of Yanbian cattle was higher than that of Yanhuang cattle, while the relative abundance of *Firmicutes* and *Bacteroidetes* was lower than that of Yanhuang cattle (Figures 4A,D,G). In addition, at the genus level, *Ralstonia* and *Stenotrophomonas* were both higher in the vagina, cervix and uterus of Yanbian cattle to a certain extent than in Yanhuang cattle (Figures 4B,E,H). Interestingly, the cervix and uterus of Yanhuang cattle also contain a relatively high abundance of *Histophilus*. The OTU of the vagina, cervix and uterus of the two breeds of cattle showed that the number of OTU from the vagina to the uterus of the two breeds of cattle increased (vagina: 3039 OTUs, cervix: 4918 OTUs, uterus: 5488 OTUs) (Figures 4C,F,I).

In order to further explore the influence of genetic factors on the diversity of reproductive tract microbiota, we compared the diversity of the same genital tract microbiota between Yanbian cattle and Yanhuang cattle. Regarding alpha diversity, as shown by the Chao 1 index (Figures 5A,E,I) and Shannon index (Figures 5B,F,J), the richness and evenness of the reproductive tract microbiota of

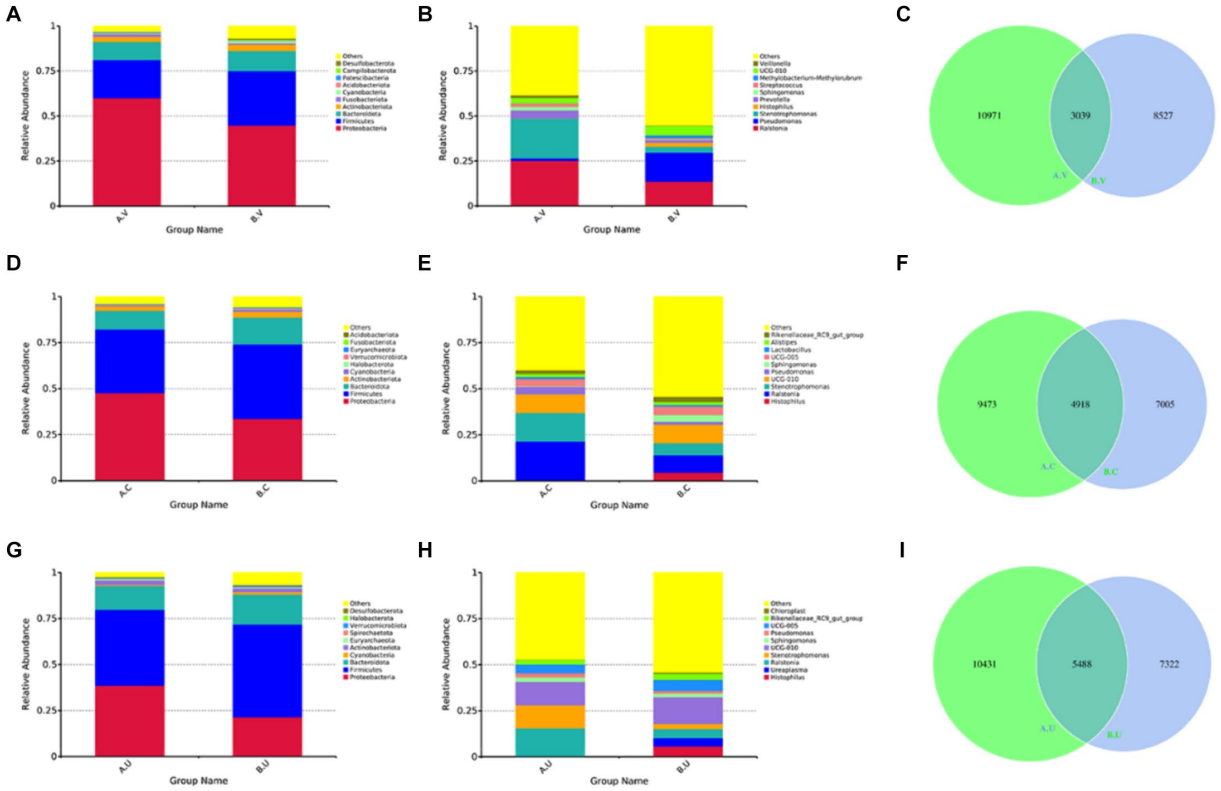

FIGURE 4
The composition and proportion of the microbiota in the same reproductive tract of Yanbian cattle and Yanhuang cattle. **(A,B,D,E,G,H)** Are the species annotations and abundance information at the phylum and genus level in the same reproductive tract of Yanbian cattle and Yanhuang cattle. Select the top 10 species with the highest abundance to generate a species relative abundance column. It is a cumulative graph. The abscissa (group name) is the group name; the ordinate (relative abundance) represents the relative abundance; others represents the sum of the relative abundance of all other phyla except the 10 phyla in the graph. Each circle in the figures **C,F,I** represent a group. The number in the overlapping part of the circle and the circle represents the number of OTUs shared between the groups. The number in the non-overlapping part represents the number of unique OTUs of the group.

Yanhuang cattle are slightly higher than those of Yanbian cattle, but there is no significant difference ($p > 0.05$). We analyzed beta diversity using the Bray–Curtis based Wilcox rank sum test method to examine differences in microbial communities between groups, there is a large dispersion in the distribution of vaginal, cervix, and uterine samples (Figures 5C,G,K), and the differences in the microbial communities in the same parts were statistically analyzed. As shown in Figures 5D,H,L, there were significant differences in beta diversity between the vagina, cervix, and uterus of Yanbian cattle and Yanhuang cattle.

In order to further identify the differential species that affect the bacterial community structure, *t*-test was used to identify species with significant differences at the phylum and genus levels, and then clarify the next research direction. At the phylum level, the number of *Cyanobacteria* in the vagina and cervix of Yanbian cattle was significantly lower than that of Yanhuang cattle ($p < 0.05$), and the number of *Desulfobacterota* in the vagina of Yanbian cattle was significantly lower than that of Yanhuang cattle (Figures 6A,B). No phylum-level species differences were observed in the uterus of Yanbian cattle and Yanhuang cattle. At the genus level, *Delftia* was significantly higher in the vagina, cervix, and uterus of Yanbian cattle than in Yanhuang cattle; *Stenobacteria* was significantly higher in the vagina, uterus of Yanbian cattle. In Yanbian cattle. In addition, *Bacteroides* was significantly lower in the vagina, cervix, and uterus of Yanbian cattle than in Yanhuang cattle, and *Mitochondria* was significantly lower in the vagina and cervix of Yanbian cattle than in Yanhuang cattle (Figures 6C–E).

Discussion

The results of this study show that the abundance and composition of bacteria in the vagina, cervix and uterus are common and different. In addition, there were differences in the composition of microbiota in the same part between the two breeds. In this study, the top three bacterial phyla in the vagina-cervix-uterus are *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. From the vagina to the uterus, the relative abundance of *Proteobacteria* shows a gradient decreasing trend. From the vagina to the uterus, the relative abundance of *Muricobacteria* showed a gradient increasing trend, and Yanbian cattle and Yanhuang cattle showed a consistent pattern. These three bacterial phyla dominate the digestive tract and may be related to the main source of reproductive tract microbiota (Zhu et al., 2023). Previous studies on the reproductive tract microbiota of humans (Liu et al., 2022) and cattle (Clemmons et al., 2017) have

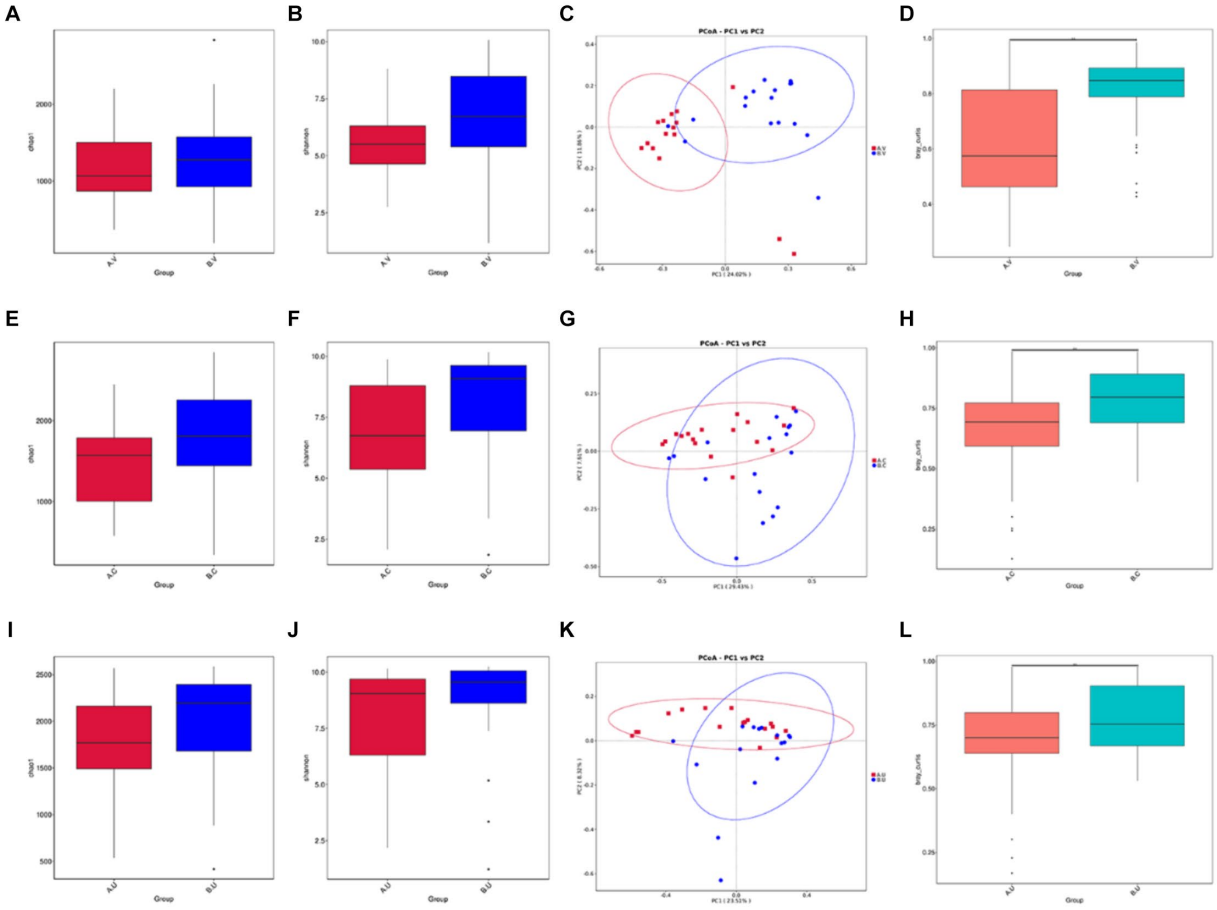

FIGURE 5
Comparison of the alpha and beta diversity of the microbiota in the same reproductive tract site of Yanbian cattle and Yanhuang cattle. Alpha diversity, including Chao 1 and Shannon index **(A,B,E,F,I,J)** of the two groups of samples, **C,G,K** based on the beta diversity of the Bray–Curtis metric of Yanbian cattle and Yanhuang cattle. The abscissa represents one principal component, the ordinate represents another principal component, and the percentage represents the contribution of the principal component to the sample difference; each point in the figure represents a sample, and samples in the same group are represented by the same color. **(D,H,L)** The Wilcox rank sum test was used to analyze the Bray–Curtis differences among each group. *Means significant difference ($p < 0.05$).

proven the dominance of these three bacterial phyla, but the proportions of each bacterial phylum are different, which may be related to environmental, nutritional and other factors. In the first three bacterial phyla, *Proteobacteria* include most of the well-known pathogenic bacteria and are potential diagnostic features of dysbiosis or disease risk (Chen et al., 2021). Studies have shown that *Firmicutes* in the digestive tract are mostly obligate anaerobic bacteria (Wozniak et al., 2022), this may be the reason for the presence of high-abundance *Firmicutes* in the anaerobic environment of the uterus. Studies have shown that the ratio of *Firmicutes* to *Bacteroidetes* is related to the occurrence of inflammation (Wu et al., 2021). Artificial control of the ratio of the bovine reproductive tract microbiota, such as the infusion of probiotics, has important potential in preventing inflammatory diseases in the bovine reproductive tract. Interestingly, we found that *Histophilus* was enriched in the reproductive tract of Yanhuang cattle, and the relative abundance increased from the vagina to the uterus. *Histophilus* is associated with several disease syndromes in cattle (Shirbroun, 2020). More recent studies indicate that *Histophilus* stimulates endothelial cell tissue factor activity and disrupts intercellular junctions (Behling-Kelly et al., 2016).

The results of the study showed an increasing trend in α diversity from the vagina to the uterus, and there are significant differences in alpha diversity between vagina and uterus, which is consistent with previous studies on human reproductive tract microbiota (Chen et al., 2017; Liu et al., 2022). However, previous studies of lactating Angus cattle have found that the alpha diversity of the vaginal flora is significantly higher than that of the uterine flora (Clemmons et al., 2017). Compared with our study, there are differences in season, region, breed, sampling method and animal feeding management mode, etc. These factors may lead to differences in the results of the two studies. From the perspective of physiological structure, the environment of tight cervix may also be an important reason for the difference in diversity of vaginal and uterine microbiota. As one of the important barriers to protect the uterine body from environmental pathogens, the cervix maintains a more stable environment of the uterus (Sheldon and Dobson, 2004; Azawi, 2008). The anaerobic environment *in utero* has a low biomass of bacteria, but corresponds to a high bacterial diversity (Chen et al., 2017). Besides, there are significant differences in beta

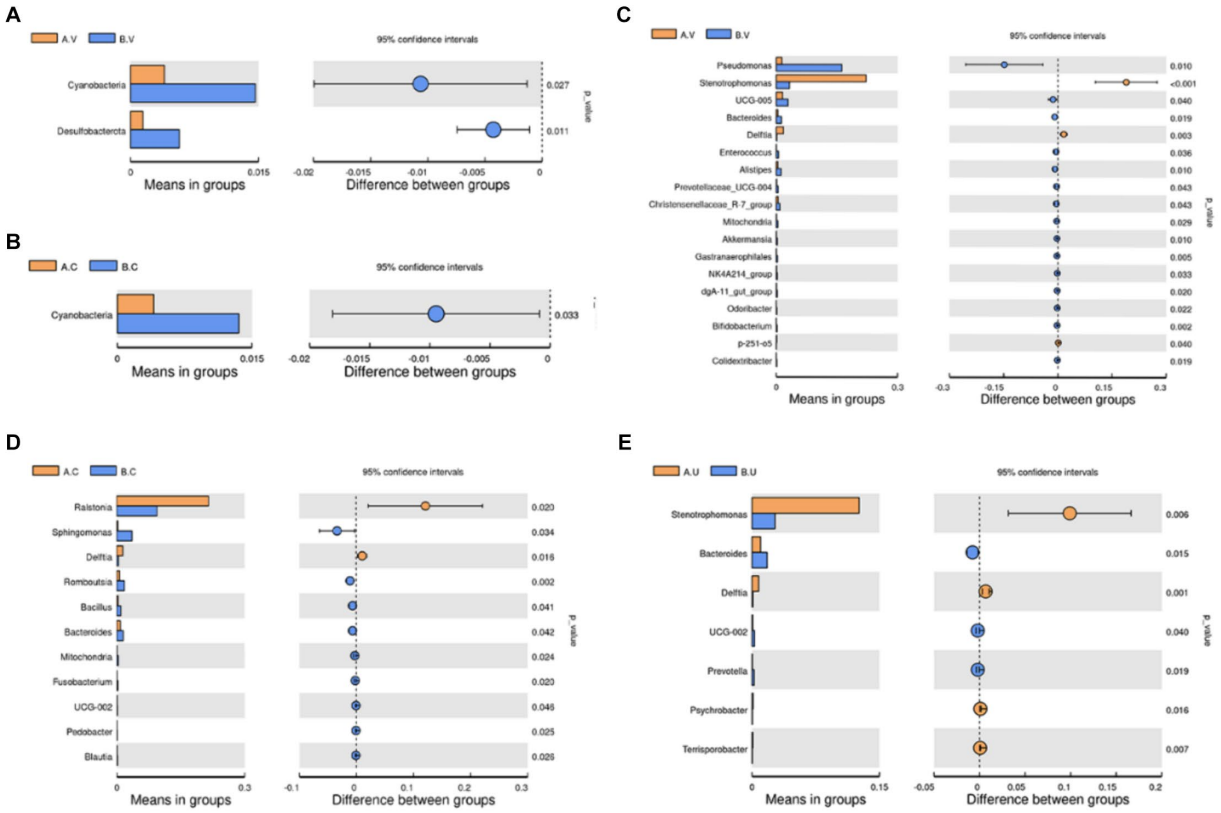

FIGURE 6
Comparison of the microbiota in the same reproductive tract of Yanbian cattle and Yanhuang cattle. Use *t*-test to identify species with significant differences between groups at the phylum **(A,B)** and genus **(C–E)** levels ($p < 0.05$). The picture on the left shows the difference in species abundance between groups. Each bar in the picture represents the mean value in each group of species with significant differences in abundance between groups. The picture on the right shows the confidence level of the difference between groups. The leftmost endpoint of each circle in the picture represents the lower limit of the 95% confidence interval of the mean difference, and the rightmost endpoint of the circle represents the upper limit of the 95% confidence interval of the mean difference. The center of the circle represents the difference between the means, and the color of the circle represents the *p*-value of the significant difference test between groups for the corresponding species.

diversity between vagina-cervix and vagina-uterus. This highlights the fact that there are differences in vaginal-cervical and vaginal-uterine microbiota profiles. *Firmicutes* were significantly enriched in the uterus of two breeds of cattle, although previous studies revealed that *Firmicutes* occupies a dominant position in the uterus (Machado et al., 2012; Santos and Bicalho, 2012; Clemmons et al., 2017), previous studies have also revealed the dominant position of *Firmicutes* in uterus. In addition, *UCG-010* was significantly enriched in the uterus of two breeds of cattle. Previous studies have demonstrated the enrichment of *UCG-010* in the intestine of cattle (Couch et al., 2021). The presence of this bacteria in the uterus may originate from the digestive tract. At the same time, the uterine environment may be more conducive to the colonization of *UCG-010*, but its role in the uterus has not yet been reported. However, *UCG-005* was only significantly enriched in the uterus of Yanbian cattle, and *Rikenellaceae_RC9_gut_group* was only significantly enriched in the uterus of Yanhuang cattle. Studies have shown that members of *Rikenellaceae_RC9_gut_group* can neutralize cytotoxic reactive oxygen species and protect cells from oxidative stress, thereby reducing the likelihood of inflammation (Gryaznova et al., 2022). We found that Yanhuang cattle only had common marker species in the uterus, and the number of marker species showed an increasing trend from vagina to uterus. This finding suggests that the uterus may have a more stable environment, making the microbiota more similar in the uterus of different species. In addition to being affected by genetic factors between breeds, the vagina and cervix are more affected by opportunistic infections in the external environment.

The research on the factor of variety shows that the relative abundance of *Proteobacteria* in the vagina, cervix and uterus of Yanbian cattle is higher than that of Yanhuang cattle, and the relative abundance of *Firmicutes* and *Bacteroidetes* is lower than that of Yanhuang cattle. The number of OTU in the uterus of Yanbian cattle and Yanhuang cattle was the highest. In previous studies, a total of 2075 OTUs were found in the vaginas of Holstein and Fleckvieh cattle (Nesengani et al., 2017), close to the results of this study. This further proves that there is a more stable environment in the uterus, making the uterus of different breeds of cattle have more similar microbiota. Previous studies have also revealed high similarities in the uterine microbiota of dairy cows in the same state (Santos et al., 2011). There is no significant difference in the alpha diversity of the vagina, cervix and uterus of the two breeds of cattle, but the alpha diversity of the microbiota in various parts of the reproductive tract of Yanhuang cattle is slightly higher than that of Yanbian cattle. The

differences in species composition between different habitats were analyzed through beta diversity. There are significant differences in the characteristics of the microbiota in the same reproductive tract of cattle breeds. This diversity difference can be explained by different breeds (Appiah et al., 2020). To this end, we further identified the species responsible for this difference at the phylum and genus level. We found that the number of *Cyanobacteria* in the vagina and cervix of Yanbian cattle was significantly lower than that of Yanhuang cattle, *Cyanobacteria* are also highly enriched in the intestines of other animals, such as yaks (Wang et al., 2021), but there is currently no scientific evidence that *Cyanobacteria* play a role in the reproductive tract. The number of *Desulfobacteria* in the vagina of Yanbian cattle was significantly lower than that of Yanhuang cattle. Although *Desulfobacteria* can participate in catabolic reactions in the intestine by reducing sulfur compounds and degrading butyrate, etc. (Bai et al., 2022). However, the specific roles played by these two bacterial phyla in the bovine reproductive tract remain to be explored. Affected by the genetic factors of the two breeds, we found differences in bacterial genera in the reproductive tracts of the two breeds of cattle. *Delftia* was significantly higher in the vagina, cervix, and uterus of Yanbian cattle than in Yanhuang cattle; *Bacteroides* is lower than that of Yanhuang cattle. *Delftia* may serve as predictor of HPV lesion evolution (Gardella et al., 2022); *Bacteroides* metabolize polysaccharides and oligosaccharides to provide nutrients, vitamins, and other functions to the host and other intestinal microbial residents (Zafar and Saier, 2021). These results indicate that the key microbiota is significantly different in the reproductive tract of Yanbian cattle and Yanhuang cattle. The role of these species with significant differences in abundance in the reproductive tract of the two breeds remains to be explored. The relationship between these differences and the reproductive traits of Yanbian cattle and Yanhuang cattle is worth further exploration.

Conclusion

In conclusion, our study found the commonalities and differences in the structure of the microbiota in different parts of the bovine genital tract, as well as the influence of breed factors on the composition of the bovine genital tract. These findings provide a solid theoretical basis for us to understand the reproductive health status of cattle, reveal the microecological balance of bovine reproductive tract, and guide the prevention and treatment of bovine reproductive diseases. The difference of reproductive tract microflora between Yanbian cattle and Yanhuang cattle reveals the possibility of microflora playing a role in the reproductive traits of the two breeds, which is worthy of further study.

Data availability statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request. All sequences used in this study are publicly available at the NCBI Sequence Read Archive under accession ID PRJNA1129596.

Ethics statement

The animal studies were approved by the Ethical Committee of Jilin Agricultural University Approval Code: 20230824001. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YT: Formal analysis, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. SF: Writing – review & editing. ZG: Formal analysis, Resources, Writing – review & editing. CH: Formal analysis, Validation, Writing – review & editing, Writing – original draft. HX: Investigation, Supervision, Writing – review & editing. ZL: Formal analysis, Writing – review & editing. JZ: Software, Supervision, Writing – review & editing. YF: Supervision, Validation, Writing – review & editing. XM: Supervision, Validation, Writing – review & editing. HL: Software, Supervision, Writing – review & editing. JG: Methodology, Supervision, Writing – review & editing. JW: Data curation, Formal analysis, Supervision, Writing – review & editing. HD: Conceptualization, Supervision, Validation, Writing – review & editing. WL: Data curation, Formal analysis, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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From pathogenesis to treatment: the impact of bacteria on cancer

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The intricate relationship between cancer and bacteria has garnered increasing attention in recent years. While traditional cancer research has primarily focused on tumor cells and genetic mutations, emerging evidence highlights the significant role of microbial communities within the tumor microenvironment in cancer development and progression. This review aims to provide a comprehensive overview of the current understanding of the complex interplay between cancer and bacteria. We explore the diverse ways in which bacteria influence tumorigenesis and tumor behavior, discussing direct interactions between bacteria and tumor cells, their impact on tumor immunity, and the potential modulation of the tumor microenvironment. Additionally, we delve into the mechanisms through which bacterial metabolites and extracellular products May affect cancer pathways. By conducting a thorough analysis of the existing literature, we underscore the multifaceted and intricate relationship between bacteria and cancer. Understanding this complex interplay could pave the way for novel therapeutic approaches and preventive strategies in cancer treatment.

KEYWORDS

cancer, bacteria, tumorigenesis, tumor prediction, therapeutic strategies

1 Introduction

As one of the most deadly and complex diseases globally, cancer has long captivated the attention of scientists, doctors, and researchers. Despite significant advancements in cancer treatment and prevention over the past few decades, the precise etiology and underlying mechanisms of cancer development remain largely enigmatic. Recently, an increasing body of research has begun to underscore the potential role of microbes, particularly bacteria, in the initiation and progression of cancer (Sepich-Poore et al., 2021). This burgeoning field presents a novel perspective on cancer pathogenesis and holds the potential to revolutionize therapeutic strategies, offering new avenues for treatment.

Traditionally, cancer research has primarily centered on the abnormal proliferation and genetic mutations of tumor cells. However, with advancements in technology and the broadening of scientific perspectives, accumulating evidence now indicates that bacteria are integral players in tumor development (Yang et al., 2023; Goto, 2022; Ghaddar et al., 2022). The relationship between bacteria and tumors is complex and multifaceted. Bacteria can directly influence tumor cell proliferation, invasion, and metastasis through direct interactions. Additionally, bacteria have the capacity to modulate the immune response within the tumor microenvironment, potentially compromising the host's immune defense against cancer and facilitating immune evasion by the tumor. Furthermore, bacterial metabolites and extracellular products can exert significant effects on tumor cells, further contributing to cancer progression.

Despite the growing body of evidence supporting the role of bacteria in tumor development, our understanding of the intricate interplay between bacteria and cancer

remains limited. Research in this domain is still in its early stages, with many critical questions yet to be addressed. A comprehensive understanding of the presence and mechanisms of bacterial influence across various cancer types, as well as their complex interactions with the host immune system, tumor cells, and diverse microbial communities, is essential for advancing our knowledge in this field. This review seeks to provide a detailed examination and synthesis of current research advancements regarding the complex association between bacteria and cancer, and to explore the potential implications of these findings for novel cancer treatment and prevention strategies.

2 The role of bacteria in cancer development and prediction

2.1 Intracellular bacteria

The traditional belief is that there are no bacteria in tumor tissue, but with the advancement of research, it has been demonstrated that bacteria primarily exist in tumor cells and immune cells in tumor tissues (Nejman et al., 2020; Livyatan et al., 2020) by using 16srDNA sequencing, QPCR, FISH, LPS, and LTA antibody fluorescence staining (Fu et al., 2022; Geller et al., 2017) and other experimental techniques (Figure 1).

Although these approaches May have limitations, including potential contamination, sensitivity to detection, and difficulty in distinguishing between live bacteria and bacterial residues, a growing body of research has shown that a wide variety of bacteria are present in different types of tumors, and that these bacteria influence tumor progression through different mechanisms. In breast cancer cells, intracellular bacteria are relatively diverse, primarily originating from three phyla: Proteobacteria, Firmicutes, and Actinobacteria. Although *Staphylococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus* are present at lower abundances, they play a crucial role in modulating the RhoA/ROCK signaling pathway. This modulation reduces intracellular mechanical stress, thereby enhancing the resistance of circulating tumor cells to fluid shear stress. As a result, the survival of these tumor cells in the bloodstream is improved, facilitating their metastatic colonization in distant organs (Nejman et al., 2020; Fu et al., 2022). Intracellular bacteria have also been identified within pancreatic cancer cells, predominantly from the phylum Proteobacteria, including the families Enterobacteriaceae and Pseudomonadaceae. These bacteria are believed to translocate retrogradely from the duodenum into the pancreas. Notably, *Mycoplasma hyorhinis*, a small, cell wall-deficient bacterium, has been detected not only in normal human dermal fibroblasts but also in mouse models of colorectal cancer and in human pancreatic ductal adenocarcinoma (PDAC) tissues. *Mycoplasma hyorhinis* confers chemoresistance to cancer cells by metabolizing gemcitabine into its inactive metabolite, 2′,2′-difluorodeoxyuridine, through its cytidine deaminase (CDD) activity. Additionally, other bacteria within the Enterobacteriaceae family May contribute to tumor resistance by modulating drug concentrations within the tumor microenvironment, thereby influencing tumor growth and metastasis (Geller et al., 2017).

Intracellular bacteria have also been identified in various cancers, including melanoma, ovarian cancer, bone tumors, and glioblastoma multiforme. In particular, Firmicutes are notably abundant in ovarian cancer, while Actinobacteria play a significant role in non-gastrointestinal tumors. In melanoma, the detected bacteria are predominantly Gram-positive (Nejman et al., 2020) and have been shown to stimulate the body's immune system to target and eliminate tumor cells through peptide presentation (Sepich-Poore et al., 2021). Understanding the presence and specific roles of these bacteria within tumor tissues could pave the way for novel therapeutic approaches, further illuminating the complex and multifaceted relationship between bacteria and cancer.

2.2 Non-intracellular bacteria

Extracellular bacteria also play a significant role in the onset and progression of tumors. Table 1 provides an overview of the distribution and functions of several common bacteria associated with tumorigenesis.

2.2.1 Oral bacteria

Many studies of the role of oral bacteria in cancer rely on observational data and microbiome analysis. While these methods provide valuable insights, they are subject to biases such as reverse causality and confounding factors, including diet and oral hygiene, that influence the composition of the microbiome. Despite these limitations, recent microbiological examinations have identified certain oral microorganisms that exhibit a notable selectivity for tumors, recent microbiological examinations of oral microorganisms from healthy volunteers and patients with oral cancer have revealed that certain bacteria, such as *Staphylococcus aureus*, *Exiguobacterium oxidotolerans*, *Veillonella parvula*, and *Prevotella melaninogenica* (Chocolatewala et al., 2010), exhibiting a notable selectivity for tumors, making them potential salivary markers for early oral cancer detection. Research indicates a positive correlation between oral bacteria and lung cancer (Zhou et al., 2022), while other studies highlight that bacteria present in dental plaque and saliva, particularly *S. anginosus*, *A. actinomycetemcomitans*, and *T. forsythia*, are significant risk factors for esophageal cancer and oral squamous cell carcinoma (Xiao et al., 2020; Kawasaki et al., 2021; Moghimi et al., 2020). Furthermore, bacteria in the root canal can promote cell proliferation and alter cancer cell biology, which May explain the carcinogenic potential of oral bacteria (Suprewicz et al., 2020).

Additionally, in conditions such as periodontitis, oral bacteria can enter the gut, disrupt the intestinal microflora, and cause an imbalance that triggers abnormal immune and inflammatory responses, ultimately leading to colorectal cancer (Ohashi et al., 2022; Koliarakis et al., 2019). Moreover, *Tannerella forsythia*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Treponema denticola*, which possess peptidyl arginine deiminase, May contribute to P53 gene mutations in normal cells, thereby increasing the risk of pancreatic cancer (Öğrendik, 2015; Michaud et al., 2013), *P. gingivalis* can migrate from the mouth to the pancreas, and in wild-type mice, repeated administration of *P. gingivalis* induces acinar ductal metaplasia (ADM), which is considered a precursor of pancreatic intraepithelial neoplasia (PanIN). Further studies found that *P. gingivalis* also accelerated the progression of PanIN to pancreatic ductal adenocarcinoma (PDAC) by altering the composition of the pancreatic microbiota, under stress conditions, *P. gingivalis* can protect cancer cells from reactive oxygen species (ROS) -induced cell death, thus promoting the development of pancreatic cancer (Saba et al., 2024).

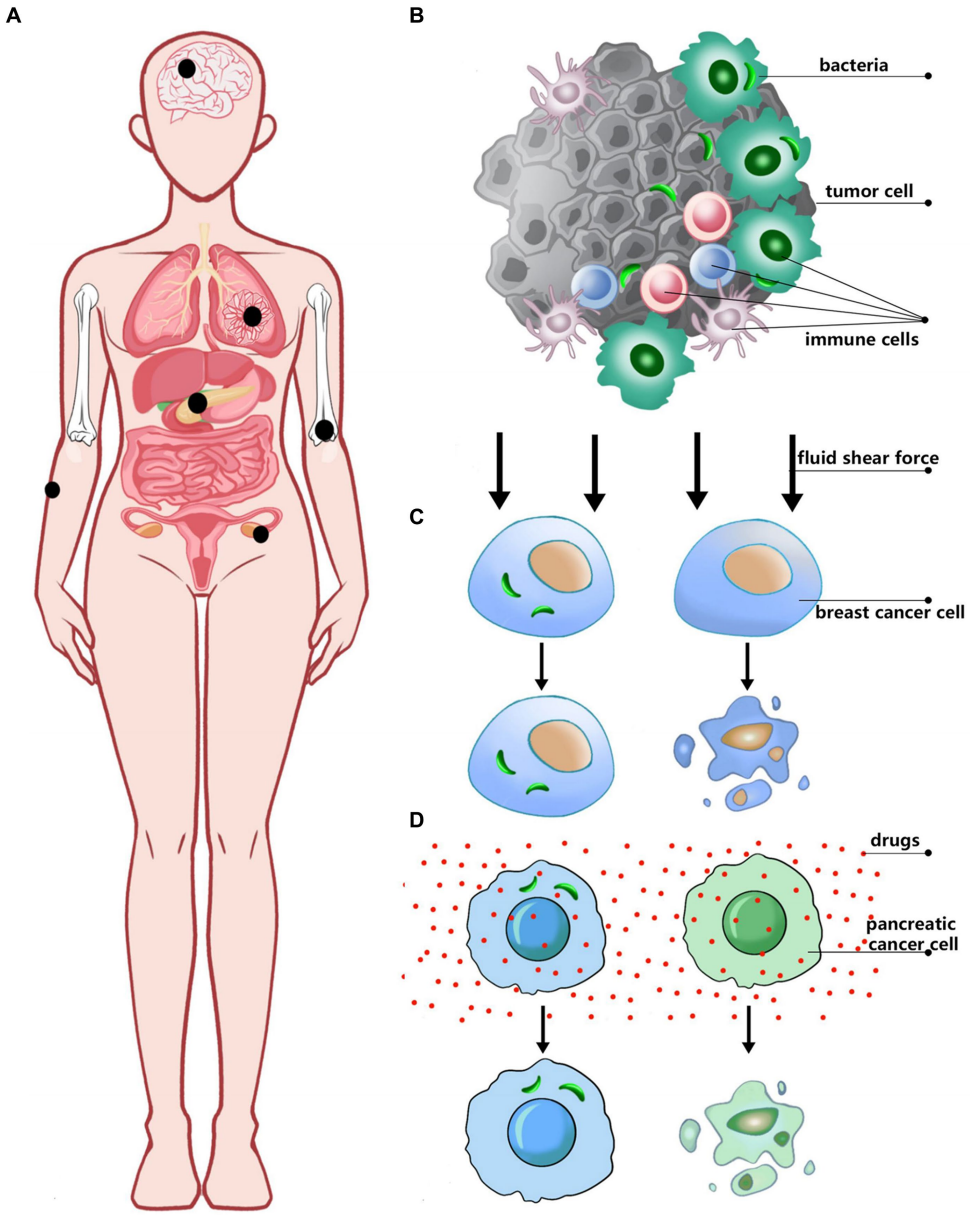

FIGURE 1
The relationship between intracellular bacteria and tumors. **(A)** Tumor types with intracellular bacteria have been discovered. **(B)** The location of bacteria in tumor tissue. **(C,D)** The role of bacteria in different cancers.

2.2.2 Gastrointestinal bacteria

The human gastrointestinal system harbors approximately 100 trillion bacteria (Quazi, 2022), which serve a crucial part in maintaining normal physiological functions. Disruption of the intestinal microbiota can lead to the overproduction of harmful metabolites and toxins, which are associated with an increased risk of inflammation and cancer. In the stomach, *Helicobacter pylori* can cause gastric mucosal epithelial cells to secrete a significant number of inflammatory mediators, increase the level of oxidative stress in these cells, and ultimately cause cancer (Zou et al., 2019). *Helicobacter pylori* can silence anti-tumor miRNAs by regulating DNA methylation at the upstream of miRNA, histone post-transcriptional modification, DNA damage and repair, and finally induce carcinogenesis (Qu et al., 2013). These mechanisms establish *Helicobacter pylori* as a major carcinogen in gastric cancer. As gastric cancer progresses, the microbiota in patients also undergoes significant changes. 16srDNA sequencing results showed that the abundance of non-*H. pylori* bacteria (such as *Proteobacteria*, *Firmicutes*, and *Actinomycetes*) was significantly increased in gastric cancer patients compared with healthy people. Although *Helicobacter pylori* remain the predominant bacterium in patients with chronic gastritis, its prevalence decreases as gastric cancer advances. Concurrently, other bacterial species, such as *Streptococcus*, *Prevotella intermedia*, and *Neisseria gonorrhoeae*, become significantly more abundant (Ferreira et al., 2018), this suggests that, in addition to *Helicobacter pylori*, other bacteria in the stomach May also contribute to the risk of developing gastric cancer (Gunathilake et al., 2021; Li and Yu, 2020; Li and Perez Perez, 2018), for instance, in mouse models, *Streptococcus anginosus* has been

TABLE 1 Cancer related microorganisms.

Microorganism	Phylum	Natural habitat	Characteristics in cancer	Related cancer	References
Staphylococcus aureus	Firmicutes	Skin, mucosa, nasal cavity, oral cavity	Being specific for tumorogenic tissues	Oral cancers	Chocolatewala et al. (2010)
Veillonella parvula	Firmicutes	Oral cavity	Being specific for tumorogenic tissues	Oral cancers	Chocolatewala et al. (2010)
Prevotella melaninogenica	Bacteroidetes	Oral cavity, respiratory tract	Being specific for tumorogenic tissues	Oral cancers	Chocolatewala et al. (2010)
S. anginosus	Firmicutes	Oral cavity, throat	Significant risk factors associated with both esophageal cancer and oral squamous cell carcinoma	Esophageal cancer and oral squamous cell carcinoma	Xiao et al. (2020), Kawasaki et al. (2021), Moghimi et al. (2020)
A. actinomycetemcomitans	Proteobacteria	Oral cavity	Significant risk factors associated with both esophageal cancer and oral squamous cell carcinoma	Esophageal cancer and oral squamous cell carcinoma	Xiao et al. (2020), Kawasaki et al. (2021), Moghimi et al. (2020)
T. forsythia	Bacteroidetes	Oral cavity (associated with periodontal disease)	Significant risk factors associated with both esophageal cancer and oral squamous cell carcinoma	Esophageal cancer and oral squamous cell carcinoma	Xiao et al. (2020), Kawasaki et al. (2021), Moghimi et al. (2020)
Tannerella forsythia	Bacteroidetes	Oral cavity (associated with periodontal disease)	Had the peptidyl arginine deaminase (PAD), which may be one of the causes of the P53 gene mutation	Pancreatic cancer	Öğrendik (2015), Michaud et al. (2013)
Porphyromonas gingivalis	Bacteroidetes	Oral cavity (associated with periodontal disease)	Had the peptidyl arginine deaminase (PAD), which May be one of the causes of the P53 gene mutation	Pancreatic cancer	Öğrendik (2015), Michaud et al. (2013)
Prevotella intermedia	Bacteroidetes	Oral cavity	Had the peptidyl arginine deaminase (PAD), which may be one of the causes of the P53 gene mutation	Pancreatic cancer	Öğrendik (2015), Michaud et al. (2013)
Treponema denticola	Spirochaetes	Oral cavity (associated with periodontal disease)	Had the peptidyl arginine deaminase (PAD), which May be one of the causes of the P53 gene mutation	Pancreatic cancer	Öğrendik (2015), Michaud et al. (2013)
Helicobacter pylori	Proteobacteria	Stomach	Secreting a significant number of inflammatory mediators; increasing the level of oxidative stress in cells, silence anti-tumor miRNAs by regulating DNA methylation at the upstream of miRNA, histone post-transcriptional modification, DNA damage, and repair	Gastric cancer, pancreatic cancer and pancreatic autoimmune inflammation	Zou et al. (2019), Qu et al. (2013), Ferreira et al. (2018), Gunathilake et al. (2021), Li and Yu (2020), Li and Perez Perez (2018)
Prevotella intermedius	Bacteroidetes	Oral cavity, possibly the gut	Increased bacterial abundance in gastric cancer patients	Gastric cancer	Ferreira et al. (2018)
Neisseria gonorrhoeae	Proteobacteria	Genital tract	Increased bacterial abundance in gastric cancer patients	Gastric cancer	Ferreira et al. (2018)
Streptococcus	Firmicutes	Oral cavity, throat, gut	Increased bacterial abundance in gastric cancer patients	Gastric cancer	Ferreira et al. (2018)
Ruminococcus gnavus	Firmicutes	Gut	Enriched in normal tissues, inhibits CD8+ T cells activity	Colorectal cancer	Zhang et al. (2023)
Blautia producta	Firmicutes	Gut	Enriched in normal tissues, inhibits CD8+ T cells activity	Colorectal cancer	Zhang et al. (2023)
Dorea formicigenerans	Firmicutes	Gut	Enriched in normal tissues, inhibits CD8+ T cells activity	Colorectal cancer	Zhang et al. (2023)
Bacteroides fragilis	Bacteroidetes	Gut	Modulating the host's metabolic pathways, immune responses, and inflammatory reactions, influencing host signaling pathways	Inflammatory bowel disease (IBD) and colorectal cancer	Chen et al. (2024), Santaolalla et al. (2013), Wu et al. (2004), Goodwin et al. (2011), Thiele Orberg et al. (2017), Wu et al. (1998)

(Continued)

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TABLE 1 (Continued)

Microorganism	Phylum	Natural habitat	Characteristics in cancer	Related cancer	References
Peptostreptococcus anaerobius	Firmicutes	Gut, skin, mucous membranes	Modulating the host's metabolic pathways, immune responses, and inflammatory reactions, influencing host signaling pathways	Inflammatory bowel disease and colorectal cancer	Chen et al. (2024), Santaolalla et al. (2013)
Clostridium septicum	Firmicutes	Gut, soil	Modulating the host's metabolic pathways, immune responses, and inflammatory reactions, influencing host signaling pathways	Inflammatory bowel disease and colorectal cancer	Chen et al. (2024), Santaolalla et al. (2013)
Parvimonas micra	Firmicutes	Oral cavity, gut	Modulating the host's metabolic pathways, immune responses, and inflammatory reactions, influencing host signaling pathways	Inflammatory bowel disease and colorectal cancer	Chen et al. (2024), Santaolalla et al. (2013)
Fusobacterium nucleatum	Fusobacteria	Oral cavity, gut,	Modulating the host's metabolic pathways, immune responses, and inflammatory reactions, influencing host signaling pathways; Induces cervical cancer progression through cytotoxic effects, immune regulation, and lipid metabolism interference	colorectal cancer; cervical cancer	Shi et al. (2016), Kitajima and Barbie (2018), Mohammadi et al. (2022), Maarsingh et al. (2022)
Prevotella	Bacteroidetes	Oral cavity, gut, urogenital tract	Enriched in endometrial cancer; associated with gene regulation (PRSS33, CPB2, XBP1) involved in fibrin degradation and cell secretion; Increased abundance linked to inflammation and lung tissue changes, contributing to NSCLC progression	Endometrial cancer, non-small cell lung cancer	Li et al. (2021), Qian et al. (2022)
Pelomonas	Proteobacteria	Various environments, including water	Promoting the development of endometrial cancer	Endometrial cancer, bacterial vaginosis	Li et al. (2021)
Lancefieldella parvula	Firmicutes	Urogenital tract	Induces cervical cancer progression through cytotoxic effects, immune regulation, and lipid metabolism interference	Cervical cancer	Maarsingh et al. (2022)
Fusobacterium gonidiaformans	Fusobacteria	Oral cavity, urogenital tract	Induces cervical cancer progression through cytotoxic effects, immune regulation, and lipid metabolism interference	Cervical cancer	Maarsingh et al. (2022)
Peptoniphilus lacrimalis	Firmicutes	Urogenital tract	Induces cervical cancer progression through cytotoxic effects, immune regulation, and lipid metabolism interference	Cervical cancer	Maarsingh et al. (2022)
Porphyromonas uenonis	Bacteroidetes	Oral cavity, urogenital tract	Induces cervical cancer progression through cytotoxic effects, immune regulation, and lipid metabolism interference	Cervical cancer	Maarsingh et al. (2022)
Escherichia coli	Proteobacteria	Gut, urogenital tract	Enhances expression of lipid synthesis enzymes (FASN, ACC1) via TLR4/TLR9 pathways, promoting proliferation and metastasis	Non-small cell lung cancer	Ye et al. (2016)
Roseburia	Firmicutes	Gut	Altered abundance associated with lung tissue changes, contributing to NSCLC progression	Non-small cell lung cancer	Qian et al. (2022)
Gemmiger	Firmicutes	Gut	Altered abundance associated with lung tissue changes, contributing to NSCLC progression	Non-small cell lung cancer	Qian et al. (2022)

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shown to promote the development and progression of gastric cancer by interacting with the Annexin A2 (ANXA2) receptor on gastric epithelial cells through its surface protein TMPC, this interaction enhances bacterial attachment and colonization, leading to the activation of the MAPK signaling pathway. Notably, the elimination of ANXA2 blocks the MAPK activation induced by *Streptococcus anginosus*, thereby inhibiting its tumorigenic effects (Fu et al., 2024). The involvement of these bacteria in the development of gastric cancer suggests their potential as biomarkers for predicting disease (Table 2). Studies have demonstrated that the analysis of tongue coating flora in healthy individuals and gastric cancer patients identified a combination of six bacterial genera—*Peptostreptococcus*, *Peptococcus*, *Porphyromonas*, *Macromonas*, *Rothia*, and *Fusobacterium*—as the most effective predictive model for distinguishing gastric cancer patients from healthy controls (Xu et al., 2021). Further microbiome analysis also revealed a significant increase in the abundance of *Oscillospira*, *Escherichia*, *Faecalibacterium*, and *Desulfovibrio* in the stool of gastric cancer patients. Notably, *Desulfovibrio* was significantly more abundant in stage IV gastric cancer patients compared to those in stages I, II, and III (Liu et al., 2021).

Intestinal bacteria are closely linked to the pathogenesis of inflammatory bowel disease (IBD) and colorectal cancer (CRC). Studies have revealed significant differences in the composition of intestinal flora among healthy individuals, IBD patients, and CRC patients (Ma et al., 2021), compared to normal individuals and CRC patients, IBD patients exhibit an increased abundance of *Bacteroides* and a decreased abundance of Firmicutes, in contrast, CRC patients demonstrate an increased abundance of Fusobacteria, Firmicutes, Verrucomicrobia, Bacteroides, and Proteobacteria (Xu et al., 2020). Further studies showed that symbiotic bacteria belonging to the Lachnospiraceae family, such as *Ruminococcus gnavus*, *Blautia producta*, and *Dorea formicigenerans*, are enriched in normal tissues, they can degrade glycerolysin, a compound that inhibits CD8(+) T cell activity. By breaking down glycerolysin, these bacteria promote the activation of CD8(+) T cells, thereby enhancing immune surveillance and inhibiting the growth of colon tumors (Zhang et al., 2023); *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* were more common in tumor tissues, among them, *Fusobacterium nucleatum* plays a multifaceted role in colorectal cancer progression. The FadA protein secreted by *Fusobacterium nucleatum* has been shown to regulate epithelial cell proliferation, contributing to tumor growth (Taddese et al., 2020). Additionally, *Fusobacterium nucleatum*-derived outer membrane vesicles (OMVs) can also significantly enhance the metastatic potential of cancer cells, specifically, in mouse models, these OMVs promote lung metastasis and increase cancer cell migration and invasion *in vitro*, the underlying mechanisms include the activation of autophagy flux and alterations in the expression of proteins associated with epithelial-mesenchymal transition (EMT) (Chen et al., 2024). Moreover, *Fusobacterium nucleatum*'s outer membrane protein contains

TABLE 2 The diagnostic potential of bacteria in different types of cancer.

Strain(s)	Type of cancer	Bacterial action	References
P. melaninogenica *Streptococcus mitis* *Capnocytophaga gingivalis*	Oral cancer	Distinct salivary specificity in oral cancer, making them potential salivary markers for early oral cancer detection	Chocolatewala et al. (2010)
Parvimonas micra *Clostridium symbiosis* *Hungatela hathewayi* *Peptostreptococcus stomatis* *Gemella morbillorum*	Colorectal cancer	Diagnostic model for patients with colorectal cancer	Zhang et al. (2022), Yao et al. (2021), Osman et al. (2021), Rezasoltani et al. (2018)
Prevotella copri *Parvimonas micra* *Parvimonas micra* *Cetobacterium somerae* *Gemella morbillorum*	Colorectal cancer	Diagnostic model for patients with colorectal cancer	Zhang et al. (2022), Yao et al. (2021), Osman et al. (2021), Rezasoltani et al. (2018)
Enterococcus faecalis *Streptococcus bovis* Enterotoxigenic *Bacteroides fragilis* *Porphyromonas* spp. *Fusobacterium nucleatum*	Colorectal cancer	Diagnostic model for patients with colorectal cancer	Zhang et al. (2022), Yao et al. (2021), Osman et al. (2021), Rezasoltani et al. (2018)
Peptostreptococcou *Peptococcus* *Porphyromonas* *Macromonas* *Rothia* *Fusobacterium*	Gastric cancer	Prediction model for distinguishing gastric cancer patients from healthy people	Xu et al. (2021), Liu et al. (2021)
Oscillospira *Escherichia* *Faecalibacterium* *Desulfovibrio*	Gastric cancer	Potential biomarkers for predicting gastric cancer	Xu et al. (2021), Liu et al. (2021)

lipopolysaccharide (LPS), a pathogen-associated molecular pattern that binds to Toll-like receptor 4 (TLR4) on the surface of host cells. This interaction initiates the TLR4 signaling pathway, leading to the activation of the myeloid differentiation primary response gene 88 (MYD88), a crucial adaptor protein that triggers the downstream NF-κB pathway. Once activated, NF-κB translocates to the nucleus, where it promotes the expression of pro-inflammatory cytokines and tumor-promoting genes such as miR-21. MiR-21, a well-known pro-inflammatory microRNA associated with colorectal cancer, inhibits the expression of RAS-GTPase activating protein family member RASA1, resulting in the sustained activation of the RAS signaling pathway and the subsequent initiation and progression of colorectal cancer (Santaolalla et al., 2013; Shi et al., 2016; Kitajima and Barbie, 2018).

Anaerobic bacteria like *Bacteroides fragilis*, *Peptostreptococcus anaerobius*, *Clostridium septicum*, *Porphyromonas gingivalis*, and *Parvimonas micra* play pivotal roles in colorectal cancer progression by modulating the host's metabolic pathways, immune responses, and inflammatory reactions (Mohammadi et al., 2022; Justesen et al., 2022). *Bacteroides fragilis*, for instance, secretes a zinc-dependent metalloprotease known as *Bacteroides fragilis* toxin (BFT), which degrades E-cadherin in epithelial cells, leading to the nuclear translocation of *β*-catenin and activation of the Wnt/β-catenin signaling pathway. Concurrently, BFT activates the p38 MAPK and NF-κB signaling pathways. Activation of p38 MAPK upregulates the expression of cyclooxygenase-2 and prostaglandin E2, both of which play critical roles in cancer cell proliferation and tumor formation. Moreover, p38 MAPK regulates Spermine oxidase (SMO), resulting in the production of reactive oxygen species, which cause DNA damage and increased cell proliferation (Wu et al., 2004; Goodwin et al., 2011). Furthermore, BFT enhances the expression of CXC chemokines via the NF-κB pathway, promoting the recruitment of inflammatory cells and sustaining cancer cell proliferation within CRC tissues (Thiele Orberg et al., 2017; Wu et al., 1998). The significant role these bacteria play in CRC progression also highlights their potential as biomarkers for cancer prediction (Table 2). For example, studies have shown that the abundance of bacteria such as *Parvimonas micra*, *Clostridium symbiosum*, *Hungatella hathewayi*, *Peptostreptococcus stomatis*, and *Gemella morbillorum* increases significantly in CRC patients, suggesting that a combination of these bacteria could serve as a diagnostic model for CRC (Zhang et al., 2022). Similarly, another study proposed a prediction model based on the presence of *Prevotella copri*, *Parvimonas micra*, *Cetobacterium somerae*, and *Gemella morbillorum* (Yao et al., 2021). In addition, *Fusobacterium nucleatum*, *Akkermansia muciniphila*, *Parvimonas micra*, and *Peptostreptococcus stomatis* have also been detected in a large number of CRC patients (Osman et al., 2021). The combination of *Enterococcus faecalis*, *Streptococcus bovis*, *Bacteroides fragilis*, *Porphyromonas* spp. and *Fusobacterium nucleatum* also showed a high diagnostic value for CRC (Rezasoltani et al., 2018).

Certain bacterial metabolites, such as butyrate, have been shown to have anti-inflammatory and anticancer effects, and can enhance the efficacy of chemotherapy drugs (such as gemcitabine) against pancreatic cancer (Tarashi et al., 2019; Panebianco et al., 2022). Butyrate inhibits the development of colorectal cancer by maintaining intestinal epithelial barrier function, inhibiting inflammation, and inducing cancer cell apoptosis. It also acts as a histone deacetylase (HDAC) inhibitor, regulating gene expression and thereby preventing the proliferation and migration of cancer cells. However, some *Porphyromonas* species also secrete butyrate, but accelerate the development of colorectal cancer by inducing cell senescence (Qu et al., 2023), this finding suggests that the same metabolite, butyrate, May play complex and context-dependent roles in cancer development depending on its bacterial source, the imbalance of butyrate levels is also shown in patients with non-small cell lung cancer, indicating that metabolites produced by intestinal flora play an important role in the occurrence and progression of cancer (Gui et al., 2020). Beyond butyrate, other bacterial metabolites also influence tumor progression. For example, indole-3-propionic acid (IPA), a metabolite derived from *Lactobacillus johnsonii* or tryptophan, can enhance the efficacy of *α*PD-1 immunotherapy mediated by CD8+ T cells. IPA further enhances the anti-tumor immune response by increasing H3K27 acetylation in the Tcf7 super enhancer region, modulating the dry program of CD8+ T cells, and promoting the production of depletion precursor CD8+ T cells (Jia et al., 2024). Additionally, certain intestinal bacteria produce androgens, which can increase both the incidence of and resistance to therapy in prostate cancer (O'Leary, 2021a), in a mouse model, infection with *Helicobacter hepaticus* through the gastrointestinal tract led to a systemic elevation of pro-inflammatory cytokines, including TNF-α, IL-1α, IL-3, and eotaxin. These mice exhibited significant increases in precursors of prostate cancer, such as prostatic intraepithelial neoplasia and microadenocarcinoma (Poutahidis et al., 2013).

2.2.3 Other aspects

The complex relationship between bacteria and cancer is not limited to the mouth and gastrointestinal tract, with new research showing that bacteria in other parts of the body are also strongly linked to the progression of cancer. For instance, microbial analysis of endometrium from patients with endometrial cancer and healthy volunteers revealed that *Prevotella* and *Pelomonas* were enriched in the endometrial cancer group, *Prevotella* was significantly associated with three genes (PRSS33, CPB2, XBP1). These genes are involved in the degradation of fibrin, the regulation of the coagulation and fibrinolysis system, and the regulation of the cell secretion system, thus promoting the progression of endometrial cancer (Li et al., 2021); bacteria associated with bacterial vaginosis, including *Lancefieldella parvula*, *Fusobacterium gonidiaformans*, *F. nucleatum*, *Peptoniphilus lacrimalis*, and *Porphyromonas uenonis* induces cervical cancer progression through direct cytotoxic effects on cervical cells, alterations in immune regulation, metabolic pathways, and interference with lipid metabolism (Maarsingh et al., 2022).

The progression of lung cancer is also linked to bacterial infections of the respiratory tract. According to the findings of Ye et al. (2016), gram-negative bacterial infection, particularly *Escherichia coli*, significantly enhances the expression of key lipid synthesis enzymes, FASN and ACC1, by activating TLR4 and TLR9 signaling pathways in NSCLC cells, this upregulation leads to increased lipid levels, thereby promoting the proliferation and metastatic potential of NSCLC cells. In NSCLC, significant alterations in the gut microbiota structure of mice have been observed. Specifically, the abundance of bacterial genera such as *Prevotella*, *Roseburia*, and *Gemmiger* increased markedly. Oral administration of *P. copri* to mice exacerbated inflammation, disrupted immune homeostasis, and led to significant structural changes in lung tissue, ultimately contributing to the progression and development of NSCLC (Qian et al., 2022).

The intricate relationship between bacteria and various cancers underscores the pivotal role of the microbiome in disease progression and treatment. These insights open new avenues for therapeutic

strategies, such as the use of probiotics and the modulation of bacterial metabolites, to reduce cancer risk and improve treatment outcome. Understanding and manipulating the microbiome could be key to developing personalized cancer therapies in the future.

3 The therapeutic potential of bacteria in cancer

As research progresses, the potential of bacteria to treat various cancers is increasingly being recognized. Here are the main ways to use bacteria to treat cancer (Figure 2; Table 3).

3.1 Effect of probiotics on cancer

Lactic acid bacteria, a well-known group of probiotics, are gram-positive microorganisms essential for maintaining the stability of the gastrointestinal flora. These bacteria and their metabolites have been shown to enhance immunity, improve antioxidant capacity, and reduce blood sugar and cholesterol levels (Liu et al., 2021). Research suggests that culturing CAco2 cells with lactic acid bacteria in combination with 5-FU, and using this approach to treat breast cancer in mice, can mitigate the adverse effects of chemotherapeutic drugs on cells without compromising their anti-tumor efficacy (Levit et al., 2021). Lactic acid bacteria have been found to reduce Stat3 expression and secrete IL-6, thereby inhibiting breast cancer stem cells, suggesting their potential in breast cancer treatment and prevention (Dwi Ningtiyas et al., 2021; Choi et al., 2018; Ohashi et al., 2002).

Beyond breast cancer, lactic acid bacteria also play a significant role in the treatment and prevention of bladder cancer, regular consumption of lactic acid bacteria has been associated with a lower incidence of bladder cancer, likely due to their ability to modulate the immune response and maintain a healthy urinary tract microbiome (Ohashi et al., 2002).

In colorectal cancer, lactic acid bacteria have been shown to significantly enhance host immunity and reduce intestinal inflammation, they inhibit cancer cell proliferation by producing volatile fatty acids, adhering to tumor cells, and reducing harmful bacteria within tumor tissue. Notably, *Lactococcus lactis* has been shown to enrich the gut microbiota with beneficial probiotics and secrete the functional protein alpha-mannosidase, which exerts significant anti-tumor effects, this protein can inhibit the growth of colorectal cancer cells and patient-derived organoids in both *in vitro* and *in vivo* experiments, leading to reduced tumor volume in mouse models (Su et al., 2024).

In gastric cancer, lactic acid bacteria inhibit the growth of *Helicobacter pylori*—a major risk factor for gastric cancer—by producing organic acids and antimicrobial substances. This inhibition reduces the risk of chronic inflammation and malignant transformation of the gastric mucosa (Hwang et al., 2012). Furthermore, long-term consumption of probiotics has been associated with a reduction in tumor size and number, as well as an increase in IL-18 production. Studies have also shown that lactic acid bacteria extracted from Kefir can enhance the cytotoxic effects of human natural killer cells on chronic myeloid leukemia and colorectal cancer cells. This cytotoxicity is mediated through the modulation of the immune system, underscoring the potential of lactic acid bacteria to enhance the efficacy of immunotherapies (Yamane et al., 2018; Riaz Rajoka et al., 2017; del Carmen et al., 2017; Hradicka et al., 2020). However, the effectiveness of lactic acid bacteria can be influenced by the strains used, the dosage, and the duration of treatment. Differences in experimental design across studies make it challenging to compare results directly, and further standardization in methodologies is needed to validate these findings across different populations and conditions.

In summary, lactic acid bacteria contribute to cancer prevention and treatment across various types of cancer, by modulating immune responses, reducing inflammation, and inhibiting tumor growth through both direct and indirect mechanisms.

3.2 The therapeutic effect of fecal microbial transplantation on tumor

Fecal microbiome transplantation (FMT) has been gaining increasing attention as a strategy to enhance the efficacy of cancer treatment by regulating the gut microbiota, which can enhance the anti-tumor effect by reconstructing the gut microbiota and enhancing the gut and systemic immune response (Lu et al., 2022; Zaman et al., 2024; O'Leary, 2021b).

FMT has demonstrated significant therapeutic potential across various tumor types, including stomach cancer, colorectal cancer, melanoma, and liver cancer (Chen et al., 2023; Doocey et al., 2022; Wang et al., 2023). By reconstituting the gut microbiota in patients, FMT can enhance antigen presentation, promote effector T cell function, and improve both the tumor microenvironment and systemic immune responses, thereby increasing the effectiveness of immunotherapies such as PD-1 inhibitors. For instance, in melanoma patients who initially do not respond to PD-1 inhibitors, the combination of FMT and PD-1 inhibitors has been shown to induce partial or complete remission. Patients who respond favorably to this treatment tend to exhibit higher α diversity in their gut microbiota, with an enrichment of *Ruminococcaceae* and *Faecalibacterium,* These microbial communities enhance immune responses both systemically and within the tumor microenvironment by improving antigen presentation and effector T cell function (Baruch et al., 2021; Gopalakrishnan et al., 2018). Additionally, studies have found that FMT from healthy donors, when combined with PD-1 inhibitors such as nivolumab or pembrolizumab, significantly increases gut microbiota diversity and improves treatment outcomes in cancer patients, over time, the gut microbiota of responding patients becomes more similar to that of the donor, with an enrichment of immunogenic bacteria and a reduction in harmful bacteria following FMT. These changes enhance the efficacy of PD-1 inhibitors by strengthening both intestinal and systemic immune responses (Routy et al., 2023; Tanoue et al., 2019). Moreover, oral supplementation with *Akkermansia muciniphila* has been shown to restore the efficacy of PD-1 blockade by increasing the recruitment of specific T cells in tumor tissue. This effect is dependent on the IL-12 signaling pathway and is achieved through the recruitment of CCR9+CXCR3+CD4+ T lymphocytes within the tumor microenvironment (Routy et al., 2018). In the treatment of castration-resistant prostate cancer, changes in the gut microbiota are also closely related to the effect of anti-androgen therapy, and

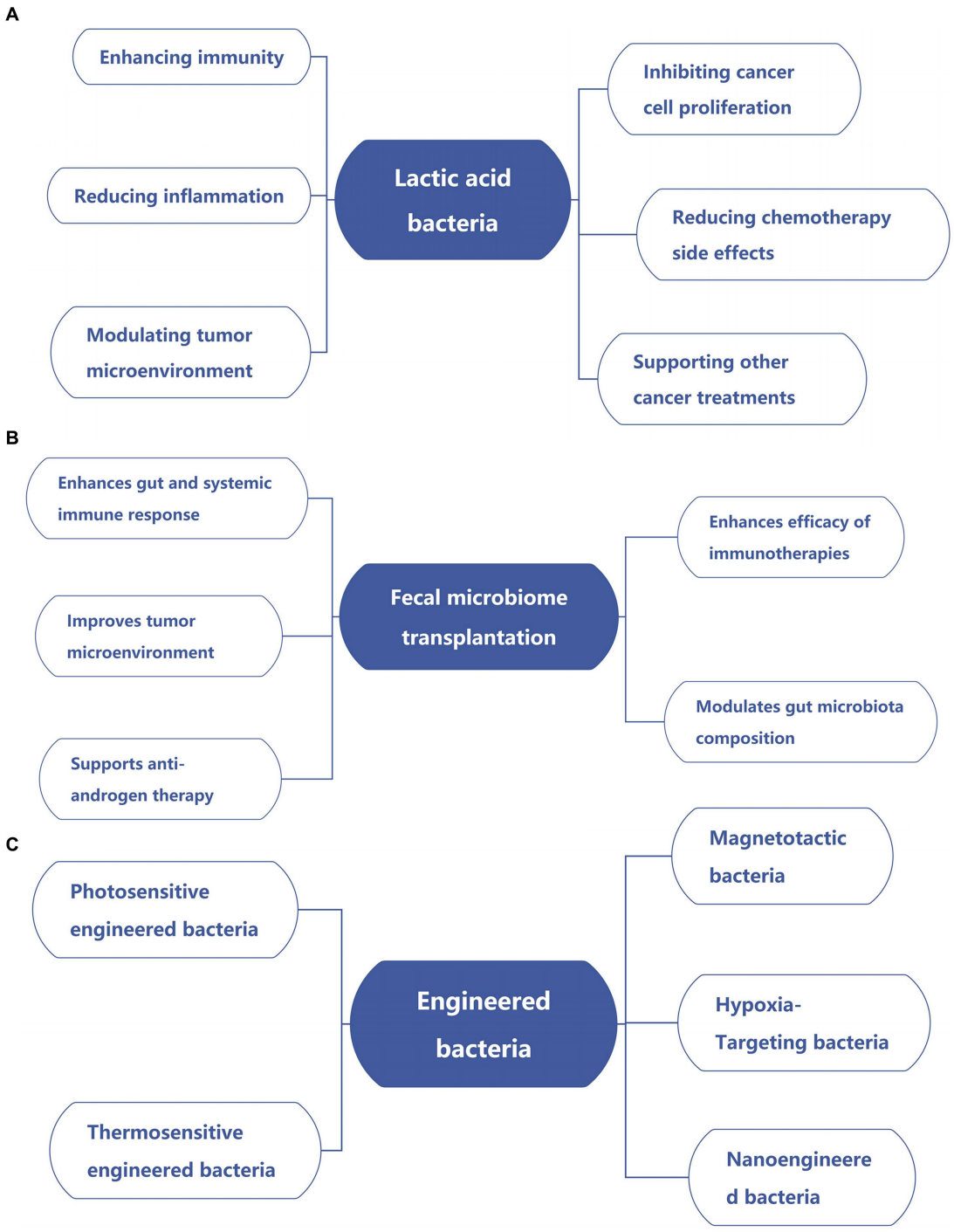

FIGURE 2
Emerging strategies for bacterial-based cancer therapies. **(A)** Lactic acid bacteria in cancer therapy. **(B)** Impact of fecal microbiome transplantation on cancer. **(C)** Applications of engineered bacteria in cancer.

FMT shows potential in delaying castration-resistant prostate cancer (Pernigoni et al., 2021). For advanced renal cell carcinoma, the composition of the gut microbiota significantly influences the efficacy of immune checkpoint blocking (ICB). Antibiotics and tyrosine kinase inhibitors reduce the efficacy of opdivo by altering the gut microbiota, particularly by increasing the abundance of *Clostridium Hathewayi*. The therapeutic efficacy of ICBs can be improved through FMT or the introduction of beneficial symbiotic bacteria, further suggesting the potential for gut microbiome regulation as a strategy for enhancing cancer immunotherapy (Derosa et al., 2020). FMT has shown promise in enhancing cancer treatment, but the methodologies used in these studies warrant careful consideration. Variability in donor selection, preparation methods, and FMT administration routes can lead to inconsistent results. Additionally, the lack of standardized protocols across studies and potential biases related

TABLE 3 Therapeutic effects of engineered bacteria in different types of cancer.

Strain(s)	Type of cancer	Bacterial action	References
Clostridium *Salmonella* *Bifidobacterium breve*	Solid tumor	Deliver therapeutic drugs directly to tumor cells	Tangney (2010), Fujimori (2008), LI et al. (2010)
Anaerobic *Bifidobacterium infantis*	Breast tumors	Transport of loaded nanoparticles into tumor cells	Xiao et al. (2022)
Non-invasive *Escherichia coli*	Plasmid transfection Genetically modify	Photodynamic therapy of tumors Carrying overexpressed genes or drugs to enhance anti-tumor effects	Deng et al. (2021)
Attenuated *Salmonella*	Solid tumor	Express and release the fluorescent protein ZsGreen, which is highly sensitive to the identification of small tumor tissue	Panteli et al. (2015)
Bifidobacterium bifidum	Hypoxic region of solid tumor	*Bifidobacterium bifidum* can be used to transfect plasmids and deliver semiconductor nanocrystals, plasmids transfecting cytosine deaminase genes can colonize hypoxic areas of solid tumors and produce cytosine deaminase, the transferred semiconductor nanocrystals can be used for tumor imaging or treatment	Min et al. (2008), Leschner and Weiss (2016)
Rhodobacter sphaeroides 2.4.1	Solid tumor	Emit near-infrared fluorescence, which aids in visualizing the interaction between bacteria and tumor tissue	Kwon et al. (2014)
Escherichia coli Nisle 1917 (EcN)	Solid tumor	Releasing flagellin B (flaB) and bind to lanthanide up-conversion nanoparticles (UCNP). UCNP emits light in the blue region, activating EcN to secrete flaB, and then it attaches to Toll-like receptor 5 present on macrophage membranes, triggering the immune response against tumor cells through the MyD88-dependent signaling pathway	Zhu et al. (2022)
Living photosynthetic bacteria (PSB)	Solid tumor	As targeted carriers for hypoxic tumor therapy	Zheng et al. (2021)
Thermal-sensitive engineering bacteria	Solid tumor	Stimulated by heat, they will produce tumor necrosis factor α (TNF-α) in the tumor to inhibit tumor growth Express programmed cell death protein (PD1) within the tumor tissue	Li et al. (2022)
Magnetotactic bacteria (MTB)	Anoxic zone of a tumor	Penetrate the interval of 3D multichannel tube sphere Inhibiting tumor cell growth through magnetotic bacteria's magnetic field swing	Kotakadi et al. (2022), Kuzajewska et al. (2020), Mokrani et al. (2010), Wang et al. (2022), Ma et al. (2023)

to donor microbiome composition highlight the need for more rigorous, large-scale clinical trials to validate the therapeutic benefits of FMT.

These clinical studies offer compelling evidence that modulating the gut microbiota can significantly enhance the effectiveness of cancer immunotherapy, particularly in patients who have not responded to conventional immune checkpoint inhibitors. These findings not only advance our understanding of the intricate interactions between microbes and the host immune system but also lay a crucial theoretical foundation for the development of novel biotherapeutic agents in the future.

3.3 Delivery, imaging, and targeted therapy of tumors

In tumor treatment, conventional therapies like radiotherapy and chemotherapy affect not only tumor cells but also healthy cells throughout the body, therefore, targeted therapies that specifically focus on tumor cells have significant potential. The tumor has a high retention and permeability effect on biologically compatible macromolecules such as liposomes, polymer-bound anticancer medicines, micelles and so on, intravenous bacterium injection has a similar effect on biological macromolecules (Fang et al., 2016), similarly, intravenous injection of bacteria can mimic this effect, making bacteria effective carriers for delivering therapeutic agents directly to tumor cells, such as *Clostridium*, *Salmonella*, *Bifidobacterium breve*, etc. (Tangney, 2010; Fujimori, 2008; LI et al., 2010). Xiao et al. (2022) introduced a biocompatible bacterial/nanoparticle hybrid platform (Bif@DOX-NPs), utilizing anaerobic *Bifidobacterium infantis* to efficiently deliver doxorubicin-loaded bovine serum albumin nanoparticles directly into breast tumors; Deng et al. (2021) genetically modified non-invasive *Escherichia coli* through plasmid transfection to exhibit catalase activity, converting H2O2 to O2 at the tumor site. Under near-infrared (NIR) light, O2 transforms into cytotoxic 1O2, enabling the destruction of tumor cells. In addition to the bacteria themselves, extracellular vesicles secreted by bacteria have also shown promise in cancer treatment. For instance, after intraperitoneal injection in mice, these vesicles can activate the inflammasome signaling pathway, inducing the secretion of interleukin-1β (IL-1β). The increase in IL-1βpromotes the production of antigen-presenting

cell precursors, thereby enhancing the immune response during tumor antigen delivery (Liu et al., 2024).

Bacteria also play a role in tumor detection. Monitoring the specific location and concentration of bacteria targeting tumor cells is essential for adjusting treatment plans and observing therapeutic effects. Current imaging techniques, such as CT scans, have limited sensitivity for early tumor detection and recurrence monitoring. Panteli et al. (2015) developed a new detection technique using an engineered attenuated *Salmonella* expressing and releasing the fluorescent protein ZsGreen, which can identify a tumor mass as small as 0.043 mm. Other studies have shown that *Bifidobacterium bifidum* can transfer semiconductor nanocrystals, specifically quantum dots (QDs), to the deep tissues of solid tumors. These QDs can be folate-bound to target tumor cells expressing the folate receptor, aiding in tumor imaging and treatment, certain bacteria can fluorescently image tumor tissue using bacterial luciferase (Lux), with light detected by a cooled charge-coupled device detector (Min et al., 2008; Leschner and Weiss, 2016). Kwon and colleagues discovered *Rhodobacter sphaeroides* 2.4.1, a novel tumor-targeting bacterial strain capable of emitting near-infrared fluorescence, aiding in visualizing bacterial interactions with tumor tissue (Kwon et al., 2014).

In the realm of targeted tumor therapy, the role of bacteria is indispensable, and engineered bacteria can further enhance therapeutic efficacy (Wu et al., 2019; Jiang et al., 2022). For example, *Escherichia coli* Nisle 1917 was designed to release flagellin B when bound to lanthanide up-conversion nanoparticles, which emit blue light to activate immune responses against tumor cells (Zhu et al., 2022); Living photosynthetic bacteria have been used as targeted carriers for hypoxic tumor therapy, leveraging their near-infrared chemotaxis and facultative aerobic traits (Zheng et al., 2021); Thermally-sensitive engineered bacteria can produce TNF-α upon heat stimulation to inhibit tumor growth (Li et al., 2022; Xu et al., 2022) and colleagues created thermally-induced bacteria, which can express programmed cell death protein 1 within the tumor tissue. When combined with laser irradiation, it can not only destroy tumor tissue but also ameliorate the immunosuppressive phenomena of the tumor microenvironment by boosting PD1 expression, biomineralizing gold nanopar-ticles (Wang et al., 2021) induce the expression of ClyA under near-infrared laser irradiation to kill tumor cells. Nanoparticles can also transport cationic antimicrobial peptides (Parchebafi et al., 2022) with anticancer and antibacterial actions into tumor cells. However, due to the harmful and adverse effects of these accumulating chemicals in the body, their therapeutic application is limited. Magnetotactic bacteria (MTB) are nanoorganisms that can be steered and propelled to the anoxic zone of a tumor using an external magnetic field (Kotakadi et al., 2022). Magnetosomes are magnetic nanoparticles synthesized by a few magnetotactic bacteria that have great potential in targeted cancer treatment (Kuzajewska et al., 2020). *In vitro* experiment proof that (Mokrani et al., 2010) MTB has the ability to penetrate the interior of 3D multicellular tumor sphere when subjected to directional magnetic field. Wang et al. (2022) created a tumor suppression method based on magnetotactic bacteria's magnetic field swing, using RGD peptide modified MTB bacteria, tumor cells can be targeted and continuous magnetic oscillation applied to their surfaces to limit tumor growth. Ma and colleagues genetically engineered magnetotactic *Escherichia coli* bacteria to achieve tumor-specific drug release and immunotherapy under the control of an alternating magnetic field. These bacteria, equipped with a heat-sensitive promoter, enabled the controlled expression of genes that release anti-CD47 nanobodies within the tumor, thereby activating an immune response. This approach demonstrated significant therapeutic effects on both primary and distant tumors (Ma et al., 2023).

These findings underscore the broad prospects of bacterial applications in tumor treatment and detection. In the future, by further optimizing bacterial carriers and imaging technologies, it is expected that more precise and efficient tumor treatment and detection can be achieved, promoting the development of personalized medicine and bringing new hope to cancer patients.

4 Conclusion and future prospects

Recent advances in cancer research have increasingly highlighted the critical role of the microbiome in influencing tumor development and therapeutic outcomes. The complex interplay between microbial communities and the host's biological systems has opened new avenues for understanding cancer biology, presenting bacteria as not only contributors to cancer progression but also as potential allies in the fight against it. As delve deeper into the relationship between bacteria and cancer, novel diagnostic and therapeutic strategies are emerging. First, the distributional changes of specific bacteria in different cancer patients present potential biomarkers for early detection. Studies have shown that the microbial communities in patients with oral cancer, colorectal cancer, and gastric cancer differ significantly from those in healthy individuals. These differences can be exploited for early screening through non-invasive methods such as saliva and fecal analysis, aiding in early cancer detection and intervention. Additionally, probiotics and their metabolites have demonstrated significant therapeutic potential in enhancing host immune function, regulating gut microbiota balance, and alleviating the side effects of chemotherapy. Probiotics not only bolster the immune response against tumors but also mitigate the toxic side effects of chemotherapeutic drugs, thereby improving patient quality of life and offering a promising adjunctive treatment approach. In addition to probiotics, FMT has shown significant therapeutic potential in the treatment of many types of tumors. By modulating the gut microbiome, FMT can enhance antigen presentation, promote effector T cell function, improve the tumor microenvironment and systemic immune response, thereby improving the efficacy of immunotherapies such as PD-1 inhibitors. Especially in patients who do not respond to traditional immune checkpoint inhibitors, the application of FMT May alter treatment response and significantly improve efficacy. However, the long-term effects and safety of FMT need to be confirmed by further research and need to be supported by larger clinical trials and data. Moreover, the use of engineered bacteria as drug carriers for targeted therapy is rapidly advancing. These bacteria can specifically target tumor tissues and release therapeutic agents within the tumor microenvironment, significantly enhancing treatment efficacy while minimizing damage to normal tissues. As bacterial carriers and imaging technologies continue to be optimized, the potential of bacteria in tumor-targeted therapy will further expand. Concurrently, the application of bacteria in

cancer immunotherapy also shows immense promise. Certain bacteria can activate the host immune system, thereby enhancing the immune response against tumors, providing a foundation for the integration of bacteria with immunotherapy, and potentially becoming a vital component of next-generation cancer treatments. The application of bacteria in tumor detection also holds great promise. Engineered bacteria can target tumor tissues and emit detectable signals, thereby improving early tumor detection rates and providing new methods for monitoring treatment efficacy. The combination of these bacteria-based imaging technologies with existing imaging modalities is expected to achieve higher sensitivity and specificity in clinical practice, optimizing treatment decisions. In summary, the application of bacteria in cancer diagnosis, treatment, and monitoring offers broad prospects, paving the way for personalized cancer therapy and potentially playing a crucial role in clinical practice.

While research on the role of bacteria in cancer holds exciting potential, numerous challenges persist in further elucidating the relationship between microbes and tumors. Initially, studies investigating the impact of bacteria on tumor development often rely on observational data and microbiome analyses, which are vulnerable to variables such as reverse causation and confounding factors, including fluctuations in diet, lifestyle, and oral hygiene. Furthermore, the identification and quantification of bacteria within tumor tissues present technical challenges. Despite the efficacy of advanced techniques like 16S rDNA sequencing, FISH, and QPCR, issues such as sample contamination, difficulties in bacterial quantification, sequencing accuracy, and sample selection remain critical factors that can undermine the reliability of results. These technical obstacles can lead to inconsistent experimental outcomes and add complexity to the research process. Moreover, although studies have demonstrated associations between specific bacteria and certain cancer types, further validation is required before these bacteria can be reliably used as markers for early detection and diagnosis. Larger, more robust studies are necessary to confirm the accuracy of bacterial profiles and to assess the generalizability of these markers across diverse populations and cancer types. Additionally, while current research has begun to uncover connections between bacteria and cancer, the underlying causal mechanisms remain largely unexplored. Many studies are limited by small sample sizes and often focus on specific cancer types or populations, leading to heterogeneity in study design. Differences in demographic characteristics, sample sizes, and analytical methods further complicate the understanding of bacteria's role in cancer. These challenges are exacerbated by the absence of standardized research protocols, underscoring the need for larger, multi-center studies to enhance the robustness of findings. Finally, significant hurdles remain in the clinical application of microbial therapies for cancer. Ensuring the long-term safety and standardization of FMT protocols is essential for broader clinical adoption. Equally important is the optimization of engineered bacteria design to ensure precise tumor targeting while minimizing systemic side effects. The successful implementation of microbial-based personalized therapies will require addressing technical, safety, and ethical considerations to ensure their effectiveness in clinical practice.

Future research should focus on the following areas: (1) Mechanistic studies on bacteria-tumor interactions: Understanding how bacteria influence tumorigenesis at the molecular and cellular levels will aid in developing new therapeutic strategies; (2) The role of bacteria in early cancer prediction: Identifying specific bacteria that can be used as predictive markers for different cancer types; (3) Application of engineered bacteria in cancer therapy: Through genetic engineering, enhancing the targeting and therapeutic efficacy of bacteria while minimizing their impact on healthy tissues; (4) The use of bacteria in tumor monitoring: Further optimizing bacterial labeling and imaging techniques to improve the sensitivity and specificity of early tumor detection; (5) Microbiome and personalized medicine: Studying the characteristics of the microbiome in different cancer patients to explore personalized treatment strategies utilizing probiotics, bacterial metabolites and FMT.

In conclusion, the intricate interactions between bacteria and cancer offer both significant challenges and exciting opportunities. A deeper understanding of these relationships could pave the way for innovative approaches to cancer prevention, diagnosis, and treatment. By integrating microbiome research into cancer biology, we May uncover new therapeutic targets and pathways, ultimately enhancing patient outcomes. Continued exploration in this field is crucial to fully harness the potential of bacteria in combating cancer, laying the groundwork for future breakthroughs in oncology.

Author contributions

JL: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft. QT: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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The microbiota: a key regulator of health, productivity, and reproductive success in mammals

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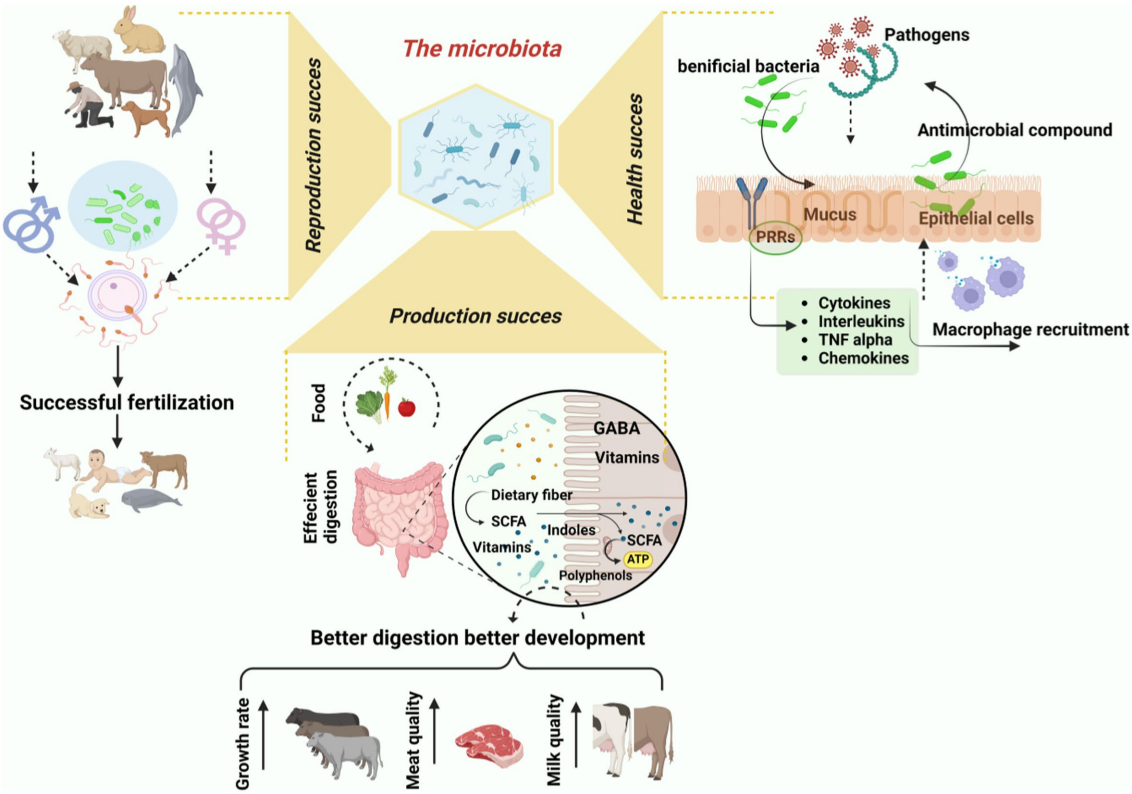
The microbiota, intensely intertwined with mammalian physiology, significantly impacts health, productivity, and reproductive functions. The normal microbiota interacts with the host through the following key mechanisms: acting as a protective barrier against pathogens, maintain mucosal barrier integrity, assisting in nutrient metabolism, and modulating of the immune response. Therefore, supporting growth and development of host, and providing protection against pathogens and toxic substances. The microbiota significantly influences brain development and behavior, as demonstrated by comprehensive findings from controlled laboratory experiments and human clinical studies. The prospects suggested that gut microbiome influence neurodevelopmental processes, modulate stress responses, and affect cognitive function through the gut-brain axis. Microbiota in the gastrointestinal tract of farm animals break down and ferment the ingested feed into nutrients, utilize to produce meat and milk. Among the beneficial by-products of gut microbiota, short-chain fatty acids (SCFAs) are particularly noteworthy for their substantial role in disease prevention and the promotion of various productive aspects in mammals. The microbiota plays a pivotal role in the reproductive hormonal systems of mammals, boosting reproductive performance in both sexes and fostering the maternal–infant connection, thereby becoming a crucial factor in sustaining mammalian existence. The microbiota is a critical factor influencing reproductive success and production traits in mammals. A well-balanced microbiome improves nutrient absorption and metabolic efficiency, leading to better growth rates, increased milk production, and enhanced overall health. Additionally, it regulates key reproductive hormones like estrogen and progesterone, which are essential for successful conception and pregnancy. Understanding the role of gut microbiota offers valuable insights for optimizing breeding and improving production outcomes, contributing to advancements in agriculture and veterinary medicine. This study emphasizes the critical ecological roles of mammalian microbiota, highlighting their essential contributions to health, productivity, and reproductive success. By integrating human and veterinary perspectives, it demonstrates how microbial communities enhance immune function, metabolic processes, and hormonal regulation across species, offering insights that benefit both clinical and agricultural advancements.

KEYWORDS

healthy microbiota, ecological function, mammalian species, production traits, immune modulation, reproductive effeciency



GRAPHICAL ABSTRACT
The graphical abstract generated with BioRender.com.

1 Introduction

The gut microbiota refers to the diverse community of microorganisms residing in the gastrointestinal tract. In contrast, the gut microbiome includes both these microorganisms and their genetic material. This intricate system is closely linked to mammalian physiology and plays a crucial role in regulating health, productivity, and reproductive functions (Dieterich et al., 2018). The human microbiota, often termed "the invisible organ," contributes over 150 times more genetic material than the human genome (Dewi et al., 2023). The composition of the microbiota varies greatly depending on its anatomical location, shaped by factors such as pH, oxygen levels, nutrient availability, and host immune responses (Lloyd-Price et al., 2016). The gut microbiota is essential for neurodevelopmental processes such as blood–brain barrier formation, myelination, neurogenesis, microglial maturation, and the regulation of animal behavior. Consequently, it is believed to play a crucial role in the development and function of the nervous system (Sharon et al., 2016). Additionally, the gut microbiota influences ovarian dysfunction and insulin resistance in polycystic ovary syndrome (PCOS) and plays a role in the neuroendocrine regulation associated with depression and obesity in humans (Milaneschi et al., 2018; Qi et al., 2019). The gut microbiota actively regulates numerous host metabolic pathways, modulates signal transduction and inflammatory responses, and serves as a vital link between key tissues and organs, including the colon, liver, muscles, and brain (Nicholson et al., 2012). Fecal microbiota transplantation (FMT) shows promising potential in veterinary medicine. It has already been used to treat gastrointestinal disorders in dogs, and ongoing research is investigating its application for conditions like ruminal acidosis in cattle and colitis in horses (Niederwerder, 2018). Further research is needed to compare microbiomes across species to better understand the specific microbial patterns linking human and veterinary medicine.

Recent studies indicate that the gut microbiota is primarily composed of several key phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, with *Firmicutes* and *Bacteroidetes* being the dominant groups

Abbreviations: GIT, Gastrointestinal tract; IVF, Invitro fertilization; PYY, Peptide tyrosine tyrosine; PCOS, Polycystic ovarian syndrome; PGN, Peptidoglycan; LPS, Lipopolysaccharide; PSA, Polysaccharide A; SCFAs, Short Chain Fatty Acids; AhR, Aryl hydrocarbon receptor; GLP-1, Glucagon-like peptide-1; AMP, Anti-microbial proteins; TLRs, Toll-like receptors; TNF, Tumor necrosis factor; DCs, Dendritic cells; TNF, Tumor necrosis factor; Th17, T helper 17; MHC, Major histocompatibility complex; IFN-γ, Interferon gamma; T6SST6SS, Type VI Secretion System; M cell, Microfold cells; PPs, Peyer's patches; FAE, Follicle-associated epithelium; GALT, Gut-associated lymphoid tissue; SED, Subepithelial dome; VFAs, Volatile Fatty Acids; IMF, Intramuscular fat; HMOs, Human Milk Oligosaccharides; THFA, Tetrahydrofolic acid; Trp, Tryptophan; AhR, Aryl hydrocarbon receptor; IBS, Irritable bowel syndrome; GLP-1, Glucagon-like peptide-1; IGN, Intestinal gluconeogenesis; Fiaf, Fasting-Induced Adipose Factor; LPL, Lipoprotein lipase; CDI, Clostridium difficile infection; GF, Germ-free; ASF, Altered Schaedler Flora; EOS, Sensitive to oxygen.

(Almeida et al., 2019; Hu et al., 2022). Emerging research has highlighted additional phyla like *Cyanobacteria and Tenericutes* that contribute to specific host interactions, emphasizing the ongoing evolution in our understanding of gut microbiota composition (Mishra et al., 2024). Further research is needed to compare microbiomes across species to better understand the specific microbial patterns linking human and veterinary medicine. Although common diseases in humans, livestock, and pets suggest shared microbial pathways, research on translating these findings across species remains limited. Firmicutes and Bacteroidetes dominate the microbiomes of many mammals, but the mechanisms governing these microbial interactions between species are still poorly understood (Laterza et al., 2016). Numerous research efforts have shed light on the crucial link between microbiota and fundamental biological functions in mammals. Recent developments, for instance, have demonstrated the significant role of human microbiota in the nutrients extraction, metabolic processes, and immune system function (Bouskra et al., 2008). Microbiota plays a crucial role in various biological processes, particularly in extracting energy and nutrients from food. This is due to its vast array of metabolic genes, which support diverse enzymes and biochemical pathways (Turnbaugh et al., 2006). In terms of the immune system, mammalian microbiota not only shields the host against foreign pathogens through the creation of antimicrobial agents but also plays a crucial role in the formation of the intestinal lining and the development of the immune system (Hou et al., 2022).

Advancements in omics-based technologies have transformed our comprehension of microbial communities associated with farm mammals and their health. The optimal functioning of the gastrointestinal tract (GIT) and its health are pivotal in influencing animal performance metrics such as body weight gain and the quality of milk and meat (Celi et al., 2017; Peixoto et al., 2021). Microbiota present in the gastrointestinal tracts of livestock and poultry break down and ferment the ingested feed, converting it into nutrients, are used to produce meat and milk (Liu et al., 2021). The symbiotic relationship between microbial communities and ruminant hosts enables the conversion of plant-based lignocellulosic biomass and non-protein nitrogen into volatile fatty acids and microbial protein. These substances are then available for the animal's growth and maintenance (Lourenco and Welch, 2022).

Sex hormones like progesterone, estradiol, and testosterone contribute to the interaction between microorganisms and their hosts, playing crucial roles in various physiological processes. These include reproduction, cell differentiation, proliferation, programmed cell death (apoptosis), inflammation, metabolism, bodily equilibrium (homeostasis), and brain functionality (Qi et al., 2021b). Changes in the microbiota, especially within the gut, can have distinct effects on the reproductive hormonal system. Rectifying imbalances in the microbiome could result in enhanced reproductive health outcomes (Franasiak and Scott, 2015). The primary role of vaginal microbiota in humans and other mammals appears to be the enhancement of reproductive success. This is achieved by offering protection against infections and contributing to immunological robustness, both crucial for the health of the endometrium, fertility, successful embryo implantation, and the overall success of pregnancy (France et al., 2022; Golińska et al., 2021; Zhang et al., 2021). Certain metabolites present in the human vagina, such as glycerophospholipids and benzopyrene, have shown a positive association with the abundance of lactobacillus and are linked to a reduced incidence of repeated implantation failures (Garcia-Garcia et al., 2022). The microbiota plays a role in the development of male reproductive organs via the gut-brain axis, enhancing the production of androgens and safeguarding the immune tolerance of the testis. Androgens maintain the balance of regulatory T cells, curb the expansion of natural killer cells, and also fortify the blood-testis barrier to shield against harmful substances (Kabbesh et al., 2021). The microbiota facilitates the growth of Sertoli cells and their intercellular connections, thus guaranteeing the creation of seminiferous tubules and preserving the integrity of the surrounding microenvironment (Cai et al., 2022).

Grasping the biological roles of mammalian microbiota is essential for understanding its critical influence on health, productivity, and reproductive characteristics, making it a focal point in research areas due to its substantial impact on host biology. In this review, we present empirical evidence demonstrating that a balanced microbiota significantly enhances the health, productivity, and reproductive capabilities of mammals. The goal of this review is to elucidate the concealed capabilities and physiological impacts of microbiota across various mammalian species, laying a theoretical groundwork for future research into leveraging microorganisms for the well-being of both humans and animals.

2 Microbial ecology across various body regions of mammals

2.1 Skin microbiota

The body skin acts as a strong physical barrier to prevent physical trauma, environmental factors, and pathogenic invasion (Schmidt, 2020). Skin is the collective habitat of bacteria, viruses, fungi, and archaea, which is become a complex ecosystem and these microorganisms are essential to skin physiology and immunity. Interactions between skin microbiota and their hosts range from mutualistic to pathogenic relationships (Apprill et al., 2010). In contrast to the more diverse microbial communities found on haired skin, the mucosal surfaces of companion animals harbor less varied bacterial populations (Kamus et al., 2018). The teat skin microbiome has also received a lot of attention, especially in relation to the diversity of microbes found in raw milk. Major taxa found upon the teat surface skin included *Staphylococcus*, *Aerococcus*, *Pediococcus*, *Pantoea*, *Enterobacter*, *Enterococcus*, and *Proteobacteria* in addition to *Corynebacteriales*, *Atopobium*, *Clostridium*, *Bifidobacteriales*, *Lachnospiraceae*, and *Coriobacteria* (Verdier-Metz et al., 2012). Also, the skin microbiomes of aquatic mammals, like humpback whales, dolphins, and killer whales, have been examined as part of marine conservation efforts. For humpback whales in different ocean regions, *Psychrobacter* and *Tenacibaculum* were identified as core genera on their skin. The abundance of these genera varied depending on the metabolic states of the whales. The Offshore bottlenose dolphins demonstrated higher skin microbial diversity compared to their coastal counterparts, whose microbiomes were influenced by coastal run off (Russo et al., 2017). The captive dolphins displayed distinct microbiomes influenced by their respective environments, particularly food and air quality. These findings emphasize the importance of wild animals in future studies focused on improving the conservation of aquatic mammals affected by skin diseases (Cardona et al., 2018).

The human skin microbiome consists of different microorganisms, and they interact with surrounding environment, such as the existence of two distinct "cutotypes" on human skin has been discovered, each associated with unique patterns of microbial networks and host skin properties (Hoffmann, 2017). The four main bacterial phyla found on the skin are *Firmicutes* (24–34%), *Proteobacteria* (11–16%), *Actinobacteria* (36–51%), and *Bacteroidetes* (6–9%) (Byrd et al., 2018; McLoughlin et al., 2021). The dry regions (e.g., hypothenar palm and volar forearm) of the skin display diverse colonization patterns among the four phyla, showcasing the highest level of diversity (Lunjani et al., 2019; McLoughlin et al., 2021; Rozas et al., 2021). Increased levels of the phyla *Proteobacteria*, *Bacteroidetes*, *Spirochetes*, *Actinobacteria*, *Firmicutes*, *Ruminococcaceae*, *Aerococcaceae*, *Corynebacteriaceae*, and *Moraxellaceae* have been linked to healthy skin (Ariza et al., 2019; Krull et al., 2014) as shown in (Table 1).

2.2 Respiratory tract microbiota

The respiratory tract including nose, nasopharynx, oropharynx, tonsils, hard plate, trachea, and lungs are contain a unique microbial community (McMullen et al., 2020). The following six *microbiome phyla*; *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, *Fusobacteria*, and *Firmicutes* could be responsible for a healthy mammals respiratory tract system (Timsit et al., 2020); however, each phylum's relative abundance and differences depending on the organ. The tonsils were colonized by *Fusobacteria*, while *Firmicutes* are widely distributed on the mouth's floor and hard palate. *Proteobacteria* are predominant in the nose, nasopharynx, and oropharynx. *Streptococcus*, *Fusobacterium*, *Mycoplasma*, *Moraxella*, and *Streptomyces* are prevalent genus along the respiratory tract, with varying distributions: *Bibersteinia* is confined to the oropharynx, *Mycoplasma* dominated the tonsils, Streptococcus dominated the floor and hard plate of the mouth, and *Mycoplasma* dominated the trachea, lung, nostril, and nasopharynx (McMullen et al., 2020).

The human respiratory tract is consisting of niche-specific bacterial communities that live there from the nostrils to the lung alveoli. Respiratory pathogen colonization is likely inhibited by the respiratory tract's microbiota, which is working as a gatekeeper. Additionally, the development and preservation of immune system and respiratory physiology homeostasis may be influenced by the respiratory microbiota. In relation to composition, the anterior nares are the most exposed to the outside world. They are lined with a keratinized squamous epithelium that resembles skin, containing serous and sebaceous glands. The latter secretes sebum, which enriches lipophilic skin colonizers such as *Propionibacterium* and *Staphylococcus* species and *Corynebacterium* spp. (Frank et al., 2010; Oh et al., 2014; Zhou et al., 2014). The anterior nares of human have also been exhibited the microbial hub including *Moraxella* spp., *Dolosigranulum* spp., and *Streptococcus* spp. that are frequently seen in other respiratory habitats (Pettigrew et al., 2012; Whelan et al., 2014; Wos-Oxley et al., 2016; Zhou et al., 2014). The stratified squamous epithelium covering the nasopharynx, which is situated deeper within the nasal cavity, is broken up by patches of respiratory epithelial cells. More species of *Moraxella*, *Staphylococcus*, and *Corynebacterium* are found in the nasopharynx's bacterial communities, which are more diverse than those in the anterior parts and show significant overlap with the anterior nares. However, other bacteria, particularly *Haemophilus* spp., *Dolosigranulum* spp., and *Streptococcus* spp. (Biesbroek et al., 2014; Bosch et al., 2016; Teo et al., 2015), are more frequently encountered in the nasopharyngeal region. The oropharynx, characterized by a non-keratinized stratified squamous epithelium, harbors a broader array of bacterial populations compared to the nasopharynx41. These encompass species are streptococcal bacteria, *Neisseria* spp., *Rothia* spp., and anaerobes such as *Veillonella* spp., *Prevotella* spp., and *Leptotrichia* spp. (De Steenhuijsen Piters et al., 2016; Segata et al., 2012; Stearns et al., 2015).

2.3 Oral microbiota

The oral cavity, encompassing the tongue, saliva, gums, tooth surfaces, buccal mucosa, and other tissues, forms a complex network that provides a highly varied territory for microorganisms, predominantly bacteria (Kilian, 2018; Lu et al., 2019). Microorganisms inhabit both the solid surfaces of teeth and the soft tissues of the oral mucosa within the diverse niches present in the mouth, creating an exceptionally intricate ecosystem. Apart from serving as the starting point for digestion, maintaining the health of the oral microbiome is essential for maintaining overall systemic health (Caselli et al., 2020). Research indicates that once children acquire their first colonizing microorganisms, the diversity of their oral microbiome expands significantly (Gomez-Arango et al., 2016). Through various bidirectional communication and regulatory mechanisms, such as microbes in the gut or mouth, work together to maintain a homeostatic balance throughout an individual's lifetime. Conversely, dysbiosis of the oral microbiota can contribute to the development of infectious diseases such as oral candidiasis, periodontal disease, and caries (Lamont et al., 2018). Given the critical role of oral health in mammals, extensive research has been conducted on the oral microbiomes of humans, as well as companion and farm animals like cats, sheep, and dogs. According to a recent report, *Burkholderia*, *Planifilum*, *Gastranaerophilales*, *Arcobacter*, *Escherichia-Shigella*, and *Actinobacteria* are the predominant genera associated with a healthy oral cavity in cattle (Borsanelli et al., 2017). The predominant bacterial phyla in the donkey oral microbiome, including *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes*, shared some similarities with the oral microbiomes of humans and other animals, albeit with slight variations. *Firmicutes*, a common phylum, was observed a common opportunistic pathogen in horse subgingival plaque and probably had been associated with periodontitis in other animal species. *Proteobacteria*, the second-highest phylum, was also present, and further investigations may shed light on its potential role in donkey periodontal diseases (Zhu et al., 2020).

A study made by Sturgeon et al. (2013) on oral microbiota in dogs, a core microbiome was identified, particularly *Porphyromonas* spp., and the association attributed to microenvironments in the dogs' oral cavities, promoting the growth of some organisms while inhibiting others. The oral microbiome in dogs displayed moderate uniformity, high diversity, and greater richness compared to the canine fecal microbiome. Another study noted that *Porphyromonas* and *Fusobacterium* were highly abundant, raising questions about their roles as supporting pathogens in dogs, particularly in dental disease (Sturgeon et al., 2013).

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TABLE 1 The relative abundance of skin microbiota in different mammalian species.

Species	Corresponding sample size	Body parts	Bacterial family	Geographic location	Biological sex	Study made by
Bos taurus	89 dairy cows	Punch biopsies of lesioned and healthy hooves	Proteobacteria, Tenericutes, Spirochaetes, Firmicutes, Bacteroidetes, Actinobacteria	New York, United States	89 females	Zinicola et al. (2015)
Sus scrofa	82 pigs sourced from Tibetan, Rongchang, and Qingyu breeds	Back skin near neck	Arthrobacter, Paenibacillus, Carnobacterium, Cellulomonadaceae, Xanthomonadaceae	Daocheng – eastern Tibetan plateau, Sichuan basin, China	Mix of boars and sows	Zeng et al. (2017)
Myotis velifer, *Myotis volans*, *Myotis californicus*, *Eptesicus fuscus*, *Tadarida brasiliensis*, *Corynorhinus townsendii*, *Antrozous pallidus*, *Parastrellus hesperus*, *Lasionycteris noctivagans*	186 bats from 13 species	Entire skin and furred region including ears, wings, uropatagia	Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Firmicutes	Arizona and New Mexico, United States	65 female and 95 males	Winter et al. (2017)
Tursiops truncatus	6 free-ranging bottlenose dolphins	Biospies	Lachnospiraceae, Gammaproteobacteria, Pseudomonas, Diaphorobacter, Acinetobacter, Acidovorax, Dechloromonas	Southern California	4 females, 2 males	Russo et al. (2017)
Felis catus	Healthy 11 and allergic 9	Healthy 12 skin spots, Allergic 6 skin spots	Alternaria and Cladosporium	Texas, United States	5 males and 6 females	Russo et al. (2017)
Myodes glareolus	157 wild bank voles	Dorsal thorax	Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Cyanobacteria	Ukraine: Kyiv and Chernobyl Exclusion Zone	63 males, 94 females	Lavrinienko et al. (2018)
Bos taurus	32 cattle	Hind limbs from abattoir	Steptococcus dysgalactiae, Treponema spp., *Klebsiella oxytoca*, *Fusobacterium necrophorum*, Pasteurella spp.	Copenhagen V, Denmark	Unidentified	Klitgaard et al. (2008)
Equus ferus	4 mares	Thorax and limb wounds had bandaged and unbandaged experimental groups	Planctomyetaceae, Acidobacteria, Fusobacteria, *Actinobacillus*	Montreal, Canada	4 mares	Kamus et al. (2018)
Canis lupus familiaris	12 healthy and 6 allergic dogs	12 skin sites (healthy), 4 skin sites (allergic)	Proteobacteria, Oxalobacteriaceae	Texas, United States	6 males, 6 females healthy; 4 males, 2 females allergic	Hoffmann (2017)
Pantroglodytes, *Gorilla gorilla*, Papio, *Macaca mulatta*	7 chimpanzees, 5 gorillas, 11 baboons, 2 rhesus macaques	Axillae	Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes	North Carolina zoo, United States.	Unknown	Council et al. (2016)
Tursiops truncates *Orcinus orca*	4 killer whales, 4 bottlenose dolphins	Dorsal, caudal, and pectoral fins; anal zone	Psychrobacter, Enhydrobacter, Staphylococcus, Sphingomonas	Antibes, France	2 males and 2 females per specices	Chiarello et al. (2017)
Lagenhorynchus obliquidens	4 Pacific white-sided dolphins	Periumbilicus skin	Pasteurellaceae, Peptostreptococcaceae, Fusobacteriaceae	Chicago, Illinois, United States	3 females, 1 male	Cardona et al. (2018)

(Continued)

TABLE 1 (Continued)

Species	Corresponding sample size	Body parts	Bacterial family	Geographic location	Biological sex	Study made by
Megaptera novaeangliae	89 humpback whales	Upper flank of dorsal spot	Psychrobacter, Moraxellaceae, Tenacibacterium, Flavobacterium	Western Antarctic Peninsula	Mix sex was collected	Bierlich et al. (2018)
Megaptera novaeangliae	56 humpback whales	Biopsy of upper flank near dorsal fin	Psychrobacter, Flavobacteria, Tenacibaculum, Gammaproteobacteria	North Pacific, North Atlantic, and South Pacific oceans	Not stated- no difference between sex observed	Apprill et al. (2014)

2.4 Gut microbiota

The gut microbiota is a highly complex and heterogeneous ecosystem, where obligate anaerobes are typically 2 to 3 times more abundant than facultative anaerobes and aerobes (Quaranta et al., 2019). The rumen is frequently characterized as a "black box" owing to the intricate diversity and complexity of its microbial ecosystem. The ruminal microbiota is recognized as a functional organ, consisting of trillions of microorganisms, with a collective metagenome that surpasses the host's genome by several hundred-fold (Human Microbiome Project, 2012). These microbial genes regulate the host's nutrition consumption and overall health via specialized metabolic pathways. As a result, the ruminal microbiota is closely connected to host feed digestion and metabolic activities. Numerous studies have shown that different groups of the ruminal microbiota have a considerable impact on feed efficiency, nitrogen digestibility, and methane production in ruminants (Schären et al., 2018). For instance, rumen methanogenic archaea primarily utilize the end products of fermentation pathways, such as hydrogen and carbon dioxide, to produce methane (CH_4) (Patra et al., 2017).

Compared to the reticulum, omasum, and abomasum, the adult rumen plays the most crucial role in the degradation of dietary organic matter due to its diverse microbial population. Rumen microbes ferment dietary carbohydrates into volatile fatty acids (VFAs), which supply up to 80% of the total energy needed by ruminants (Liu et al., 2021). Some rumen microbes also synthesize their own proteins for growth, known as microbial crude protein (MCP), by utilizing energy and nitrogen derived from the feed. Once produced, MCP is digested in the small intestine and absorbed by the host, thereby contributing significantly to the host's overall nutrition and health (Seshadri et al., 2018). *Bacteroidetes* is the most prevalent phylum in the rumen, and following by phylum Firmicutes. Moreover, the genera *Dialister*, *Succiniclasticum*, *Ruminococcus*, *Butyrivibrio*, and *Mitsuokella* collectively reported for over 1% of all bacterial genera present in the rumen (Myer et al., 2017). Numerous immune, metabolic, and nutrient absorption processes are essential to the host's survival which mediated by the gut microbiota (Manus et al., 2017; O'Hara et al., 2020).

In non-ruminant animals such as pigs, horses, and humans, the gut microbiota is critical to a variety of physiological activities such as digestion, immunological regulation, and overall health. The microbiota is mostly found in the hindgut and ferments undigested dietary components such as carbohydrates, creating short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which are important energy sources for the host. Butyrate, for example, is particularly important in equine gut health because it promotes epithelial cell development and intestinal integrity (Koh et al., 2016). In pigs, the microbiota aids in food absorption by breaking down complex polysaccharides, proteins, and lipids, as well as generating critical vitamins including vitamin K and B vitamins, which contribute to the host's nutritional status (Rook and Brunet, 2005). Furthermore, an imbalance in the microbiota, known as dysbiosis, has been linked to metabolic disorders such as obesity and insulin resistance, particularly in non-ruminant omnivores like humans and pigs, underscoring the microbiota's role in energy metabolism and disease prevention (Cani and Delzenne, 2009). Altogether, the gut microbiota in non-ruminants is integral to maintaining health, regulating metabolism, and preventing disease. In human, the gut microbiota is predominantly composed of *Firmicutes* and *Bacteroidetes*, accounting for over 90% of the population. Phyla, such as *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, play a lesser role. In addition, *Spirochetes* and *Lentisphaerae* are present in smaller quantities. The gut microbiota also hosts various other microorganisms, including archaea, yeasts, fungi, viruses, and protozoa, although their composition remains uncertain (Carding et al., 2015).

2.4.1 Small intestinal microbiota

Nonetheless, the role of the mammalian small intestinal microbiota in mediating the interactions between microbes and food is not yet fully understood. The host's ability to adjust the dietary lipid variations depends on small intestine bacteria, which regulate the gut epithelial mechanisms involved in their digestion (Martinez-Guryn et al., 2018). The small intestine, which consists of the duodenum, jejunum, and ileum, serves as the primary site for nutrient absorption. Notably, it efficiently absorbs proteins and carbohydrates from the ingested food. Furthermore, within these intestinal compartments, intricate microbial ecosystems play essential roles in processes such as fermentation, vitamin synthesis, and immune modulation (O'Hara et al., 2020). It's interesting to note that, exception of the jejunum, where proteobacteria predominated, the phylum *Firmicutes* dominated all other parts of the gastrointestinal tract in cattle. The jejunum enriched in *Acetitomaculum*, *Lachnospiraceae*, and *Ruminococcus*, whereas *Enterobacteriaceae* were highly abundant in the small intestine (Mao et al., 2015). The Firmicutes phylum had a sharp increase in relative abundance, reaching up to 80% of relative abundance, while the phylum Bacteroidetes significantly decreased (0.4:1.1%) in comparison to the rumen. There have also been published studies using low abundance phyla of *Proteobacteria* (0.8:5.8%), *Actinobacteria* (6:13%), and *Tenericutes* (0.4:4%). In addition, several other genera that are important for the small intestine are *Butyrivibrio*, *Ruminococcus*, *Mogibacterium*, *Mitsuokella*, *Propionibacterium*, *Lactobacillus*, and *Bulleidia* (Myer et al., 2017).

2.4.2 Large intestinal microbiota

The large intestine plays a vital role in absorption of water, vitamins, electrolytes, and other nutrients (Scarpellini et al., 2015). Distinct sections of the large intestine exhibit varying microbial richness and abundance in their respective microbiota. In the cecum, *Firmicutes* emerge as the predominant phylum, constituting on 70–81% of all phyla, while *Bacteroidetes* comprise the remaining 18–26%. There have also been reported of *Actinobacteria*, *Tenericutes*, and *Spirochetes* in the cecum. Moreover, the most prevalent genera in the cecum have been found to be *Prevotella*, *Coprococcus*, *Dorea*, *Ruminococcus*, *Blautia*, *Turicibacter*, *Clostridium*, and *Oscillospira* (Myer et al., 2017) and they were the most prevalent genera (Myer et al., 2017; Myer et al., 2015). In a similar vein, the phylum *Firmicutes* has also taken control of the rectum. In addition, *Roseburia*, *Osillospira*, *Clostridium*, *Bacteroides*, *Succinivibrio*, *Ruminococcus*, *Prevotella*, *Blautia*, *Turicibacter* and *Coprococcus* were the genera that dominated the rectum (Durso et al., 2017).

2.5 Genital tract microbiota

Reproductive efficiency significantly influences health and homeostasis, as well as the overall productivity of mammals. From that vantage point, it becomes imperative to comprehend the microbiome of the reproductive tract (Manes et al., 2010). The oocyte's ability to fertilize and its subsequent quality are directly influenced by the environment in which it grows. There has been inconsistent information about the presence of a microbiota in the reproductive tract. In human follicular fluid, some scientists have found cells and nucleic acids of bacteria (*Lactobacillus* spp., *Cutibacterium* spp., and *Actinomyces* spp.), but they have also documented changes between the right and left ovaries of the same host (Borges et al., 2020; Pelzer et al., 2011; Pelzer et al., 2013). However, regardless of the type of cyst and the presence or absence of endometriosis, a recent well-controlled study was unable to identify any particular microbiotas in ovarian cystic fluid (Oishi et al., 2022). The composition of the microbiota associated with follicular fluid has been successfully linked to pregnancy outcomes, even though its actual existence needs to be confirmed. Both healthy and infertile women showed a positive correlation between the presence of *Lactobacillus* spp., in the follicular fluid and the pregnancy rate following IVF and embryo transfer (Pelzer et al., 2013). Within the oviduct, crucial processes like fertilization, sperm capacitation, and early embryo development occur as part of a complicated signaling cascade. Limited information is available about the microorganisms that may inhabit or transit through the oviduct, despite the potential for interesting interactions between gametes and non-pathogenic oviductal bacteria. Semen typically contains a rich and diverse microbiota, which is important to note when discussing the male reproductive system (Cojkic et al., 2021; Farahani et al., 2020; Koziol et al., 2022; Wickware et al., 2020). Indeed, the bacterial communities found in oviducts appear to be similar to those found in semen (e.g., *Enterococcus* spp., *Cutibacterium* spp., and *Staphylococcus* spp.,) or the human vagina (*Lactobacillus* spp.,; Pelzer et al., 2018). Furthermore, it has been observed that the bacterial profiles exhibit variations in the fimbria and proximal oviduct (Brewster et al., 2022), the right and left oviducts, as well as the isthmus and ampulla (Pelzer et al., 2018). Thus far, no correlation has been found between these profiles and ovarian follicular or luteal status. In addition, menopause and hormone treatments can have an impact on the oviductal microbiota (Brewster et al., 2022). The endometrial immune system plays a crucial role in facilitating implantation and supporting fetal development, both of which are essential processes dependent on the uterine environment. According to a number of authors, the microbiota in the uterus appears to be distinct from that found in the vagina and is site-specific (Ichiyama et al., 2021; Lyman et al., 2019; Wang et al., 2021). The endometrial microbiome typically demonstrates higher bacterial variety and richness than the vagina and cervix in a wide range of animal taxa, including humans, giant pandas, dogs, domestic cattle, and horses (Diaz-Martínez et al., 2021; Ichiyama et al., 2021). The distinct bacterial genera inhabiting various body organs play pivotal roles in mammalian health, as illustrated in Figure 1, which highlights the predominant bacterial genera found in the skin, oral cavity, gut, respiratory tract, upper and lower uterine tracts, and testis, along with their emerging biological roles.

2.6 Cross talk between gut and reproductive microbiota

The gut microbiota plays a significant role in regulating reproductive hormones, which are essential for successful conception, gestation, and maternal–infant bonding. One of the key hormones influenced by gut microbiota is estrogen, which is vital for ovarian function and menstrual regulation. Studies have shown that the gut microbiota is responsible for the deconjugation of estrogens through enzymes like β-glucuronidase, which play a crucial role in regulating circulating estrogen levels. β-glucuronidase cleaves conjugated estrogens, converting them back into their active forms, which can then be reabsorbed into the bloodstream. This process is significant for reproductive health as elevated or imbalanced estrogen levels have been linked to various conditions, including endometriosis, polycystic ovarian syndrome (PCOS), and estrogen-dependent cancers such as breast cancer. Thus, understanding the activity of β-glucuronidase in the gut can provide insights into the modulation of estrogen-related health issues (Kumari et al., 2024). Dysbiosis can lead to reduced estrogen levels, which has been associated with reproductive disorders such as polycystic ovary syndrome (PCOS) and infertility (Baker et al., 2017). Additionally, gut microbiota influences other hormones crucial for reproductive health, such as progesterone and serotonin, both of which play roles in mood regulation and the establishment of pregnancy.

During pregnancy, the gut microbiota undergoes significant changes that prepare the mother for increased energy and nutritional demands. The alterations in microbial composition during the trimesters have been linked to the metabolic and immunological adaptations necessary for sustaining pregnancy and supporting fetal development (Koren et al., 2012; Nuriel-Ohayon et al., 2016). For instance, a study highlighted that specific bacterial genera increase during pregnancy, which may help modulate the immune response and reduce inflammation, supporting maternal health (Koren et al., 2012). Additionally, the microbiota may also play a role in maternal–infant bonding through the transfer of beneficial microbes during childbirth and breastfeeding, which can shape the infant's developing microbiome. As, the way of delivery significantly impacts the infant's initial microbial colonization, with vaginal births providing direct

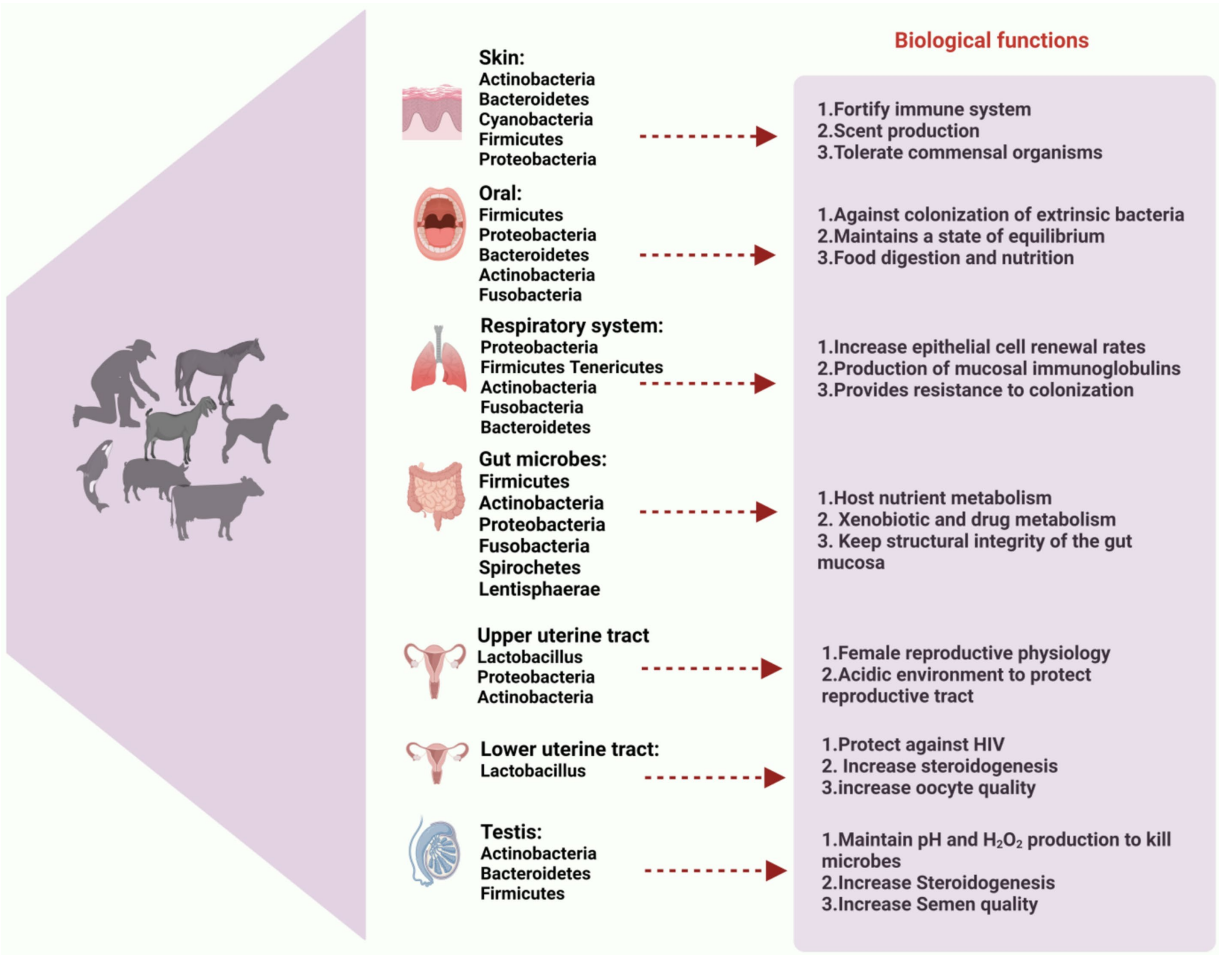

FIGURE 1
Composition of microbiota in different body organs. Predominant bacterial genera of skin, oral cavity, gut, respiratory tract, upper and lower uterine tract and testis are described and its emerging and biological role to health of mammals. This figure highlights the essential role of microbiota in maintaining overall health and homeostasis across various physiological systems. The figure created with BioRender.com.

exposure to maternal microbiota that is essential for developing a robust immune system. In contrast, infants delivered via cesarean section often miss this critical microbial exposure, potentially affecting their health and their bonding with their mother (Rautava et al., 2012). Also, breastfeeding plays a crucial role in shaping the infant's gut microbiota, as breast milk contains prebiotics and probiotics that foster the establishment of a healthy microbial community. This early microbial exposure is critical for establishing a healthy immune system and may enhance the emotional connection between mother and infant through the hormonal and biochemical signals modulated by these microbes (Bäckhed et al., 2015b; Pannaraj et al., 2017; Rautava, Luoto, et al., 2012).

Furthermore, the oral microbiota also changes during pregnancy, which has implications for maternal–infant bonding (Catassi et al., 2024). Research indicates that hormonal fluctuations during pregnancy can lead to changes in the oral microbiome, increasing the risk of conditions like gingivitis (Borgo et al., 2014; De Souza Abreu Alencar et al., 2016). This connection between oral health and hormonal changes suggests that a balanced microbiota could contribute to healthier pregnancies and possibly enhance maternal–infant bonding by reducing the risk of oral infections. Finally, emerging research points toward the potential of modifying the gut and oral microbiota to improve reproductive outcomes. Interventions aimed at restoring microbiota balance might help reduce inflammation and oxidative stress, enhancing fertility and maternal health. Future studies should focus on understanding these relationships and exploring therapeutic approaches to optimize microbiota health before and during pregnancy.

3 Mechanisms of interaction between host and microbiota

3.1 Host physiology

As a barrier, the microbiota produces substances that improve mucus production, tight junctions within epithelia, wound healing, and stem cell proliferation. These elements guarantee that the contents of the intestine remain contained. Reduced barrier function allows microorganisms or their byproducts to leak into the body and

improperly enter systemic circulation, frequently changing the inflammatory milieu (Zheng et al., 2020). Certain microbes produce surface metabolites or chemicals that can influence immune pathways, either promoting tolerance or triggering inflammation. The brain, heart, lymph nodes, or pancreas may all be affected systemically by these metabolites (Zheng et al., 2020), or they may act locally at sites where these microbes reside, such as the skin (Byrd et al., 2018), intestine, lung (Zhang et al., 2020), and mouth (Willis and Gabaldón, 2020). Additionally, the microbiota prevents the growth of pathogenic organisms that could cause or worsen disease by competing for nutrients or producing toxic and harmful metabolites (Ducarmon et al., 2019). Microbes that reside in different tissues have the capacity to produce molecules which have an immediate effect on the growth and functionality of cells (Burns and Guillemin, 2017). It is commonly discovered that microbial products affect host processes components of the outer membrane. The cell walls and outer membranes of microbes contain some of the most widely known elements and used for communication by the organisms. These communication molecules are among the most prevalent microbial products in the gut and frequently come into direct contact with host tissues. Peptidoglycan (PGN), for example, is a common component of all bacterial membranes and triggers a variety of immune signaling cascades (Zheng et al., 2020). Similarly, gram-negative bacteria's cell wall contains a significant amount of lipopolysaccharide (LPS), which is also a strong systemic immune activator (Zheng et al., 2020). Highly immunostimulatory flagellins are also widely expressed in many different bacterial taxa. However, these compounds can also encourage immunological development and tolerance. For instance, balancing the immune cell populations in the gut is facilitated by the capsular polysaccharide polysaccharide A (PSA), which is presents by the commensal *B. fragillis*. These relatively common molecules are recognized by Toll-like receptors on various host tissues, along with numerous others and they alter host physiology both locally and systemically (Zheng et al., 2020). Numerous metabolites are produced by a diverse and healthy microbiota, and these metabolites have a variety of effects on host signaling pathways. As like the tryptophan metabolites, secondary bile acids, and SCFAs are the main types of metabolites with broad effects. Acetate, butyrate, and propionate are the SCFAs that are produced when dietary fiber ferments. SCFAs typically lead to positive host outcomes, including decreased rates of obesity and diabetes, increased tolerance to immunological stimuli, and even improved brain development (Cryan et al., 2019; Thomas and Jobin, 2019). Bile acids secreted by the liver into the gut can be broken down by certain bacteria. These secondary bile acids can affect the host in a number of ways, but they are most notable for their role in endocrine signaling that affects the liver-gut disease axis and metabolic homeostasis (Molinero et al., 2019). Indole, one of tryptophan's metabolic byproducts, has been shown to have effects on hormone secretion, neurotransmitter expression, inflammation, and barrier function as in (Figure 2; Zheng et al., 2020).

3.2 Host immune system modulation

The immune response of host, which influences the susceptibility to disease, is significantly regulated by gut microbes and their metabolites (Hou et al., 2022). This happens via regular mechanical ways: Like, epithelial cells produce a number of anti- microbial proteins (AMP) and these peptides belong to the defensins, cathelicidins, and histamins families (Henrick et al., 2021). Secondly, IgA secreted by B-cells or plasma cells and recognize the microbial entry to the host (Al Nabhani et al., 2019; Chin et al., 2021). T-cells, also modulate the immune system like B-cells and become the part of the adaptive immune system, hence educated immune cells during early development to recognize self-antigens (Wang et al., 2019). The interplay between the gut microbiota and the immune system is essential for preserving host health, as the mucosal immune system acts as the primary defense against invasive gut microorganisms. Immune response elements such as tight junction proteins, antibacterial proteins, and a dense layer of mucus classify the mucosal surfaces. Innate immune cells in the gut develop tolerance to commensal bacteria by recognizing invasive pathogens and preventing their passage from the gut lumen into blood circulation (Wang et al., 2019). Upon breaching the epithelial barrier, invading bacteria and pathogen-associated molecular patterns (PAMPs), such as LPS, which swiftly reconstitute the inner mucous layer (McGuckin et al., 2011). PAMPs can induce the production of mucin from goblet cells, and they can also activate Toll-like receptors (TLRs) on neutrophils and macrophages, triggering innate immune responses (Mogensen, 2009). Additionally, commensal bacteria can activate TLRs, guiding the innate immune system to differentiate between pathogenic and commensal microbes by stimulating dendritic cells (DCs) through antigen presentation (Minarrieta et al., 2016) as presented in (Figure 3). Under normal conditions, mucosal innate immune cells, such as dendritic cells (DCs) and macrophages, engulf and eliminate invading microbes through phagocytosis (Levy et al., 2017). Furthermore, a recent discovery highlighted that the gut microbiota triggers the secretion of tumor necrosis factor (TNF) by monocytes and macrophages, facilitating the maturation of precursors type 1 conventional DCs (Köhler et al., 2020). In support of gut innate immunity, specialized epithelial cells, like goblet cells and Paneth cells, release various antimicrobial substances, including mucins, defensins, lysozyme, secretory phospholipase A2, and cathelicidins. These cells serve as supplementary immune cells alongside macrophages, neutrophils, and DCs (Johansson and Hansson, 2016). The interaction between the adaptive immune system and gut microbiota serves as a preventive measure against bacterial translocation and infection. This is demonstrated and observed that gut adaptive immune system is suppressed in germ-free mice, and the introduction of commensal bacteria can foster the development of mucosal lymphocytes, including cytotoxic CD8+ T cells and CD4+ T cells (Suzuki et al., 2010). The antigen-presenting cells, prime CD4+ T cells, and their signaling is crucial for both the primary and secondary phases of cytotoxic CD8+ T cell immunity (Bedoui et al., 2016). CD8+ T cells eliminate intracellular pathogens like *Salmonella* through antigen presentation mediated by dendritic cells (DCs) (Belz et al., 2005). The Transient Microbiota Depletion-boosted Immunization model (Becattini et al., 2020) offers a gateway to temporarily suppress microbiota-mediated colonization resistance, enabling the study of the role of tissue resident memory CD8+ T cells in preventing re-infection instances. Notably, Th17 cells induced by *Citrobacter* spp., exhibit pro-inflammatory characteristics, while Th17 cells stimulated by segmented filamentous bacteria (SFB) are non-inflammatory (Omenetti et al., 2019). Studies have revealed that germ-free mice lack Th17 cells, activated by specific microbes like SFB (Ivanov et al., 2009) and other commensal bacteria (Tan et al., 2016). It is uncovered that cytokine signals, including IL-6,

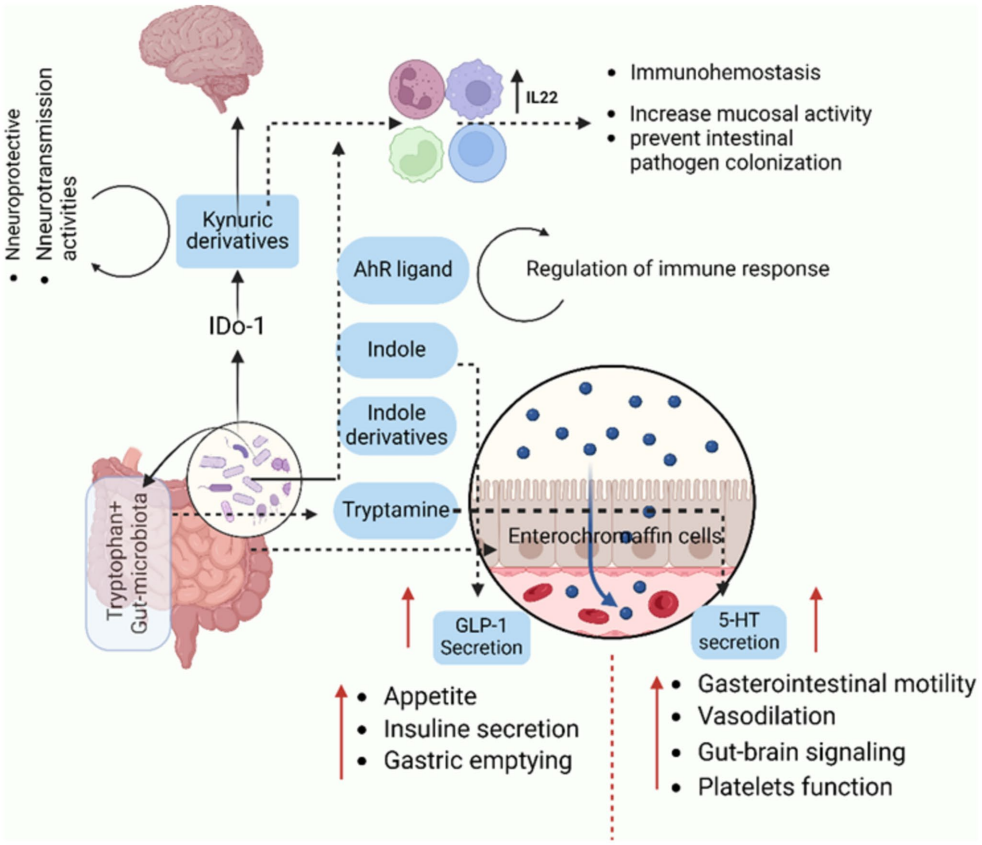

FIGURE 2
This sketch highlights how gut microbiota utilize tryptophan to produce metabolites that significantly influence physiological processes, including immune modulation, gut-brain communication, and appetite regulation. Understanding these pathways provides insights into the microbiota's role in maintaining immune homeostasis, regulating gut physiology, and even affecting neurological and metabolic health. It offers potential therapeutic strategies targeting microbiota or tryptophan metabolism for disorders related to immunity, gut health, and brain function. The metabolism of tryptophan by gut microbiota involves several pathways that lead to the production of various metabolites. Gut microbiota can convert tryptophan into indole and its derivatives have been implicated in various physiological processes, including modulation of immune responses. Indole derivatives can activate the aryl hydrocarbon receptor (AhR) and it plays a role in immune modulation. Gut enterochromaffin cells can convert tryptophan into serotonin (5- HT), influencing gut physiology and other functions and also, influence the release of GLP-1 (glucagon-like peptide-1). Both GLP-1 and serotonin are involved in the regulation of appetite and satiety. Related to kynurenine Pathway, the kynurenine pathway can be used to metabolize tryptophan, producing a number of metabolites, including kynurenic acid, which is implicated in immunological modulation and has been linked to neurotransmission disorders. The figure created with BioRender.com.

guide SFB-mediated IL-17 stimulation (Sano et al., 2021). In addition, the gut microbiome can impact Th17 responses; as investigation suggests that α2,6-sialyl ligands regulate microbiome-dependent Th17 inflammation, and α2,6-sialyltransferase deficiency triggers mucosal Th17 responses (Irons et al., 2022). Within the gastrointestinal tract (GIT), regulatory T cells (Treg) constitute an additional category of adaptive immune cells that play a role in immune tolerance. Natural Treg cells are generated in the thymus during early life through the action of an autoimmune regulator, promoting self-tolerance (Malchow et al., 2016). Subsequently, peripheral or inducible Treg production is initiated through exposure to diet and the microbiota (Ramanan et al., 2020). The gut microbiota can stimulate Treg cells in various ways. For instance, to maintain immune tolerance in the intestine, ILCs can opt for RORγt + Treg cells that specifically target the microbiota, inhibiting the proliferation of Th17 cells (Lyu et al., 2022). Immunological responses mediated by RORγt + Treg cells can also be induced by *Helicobacter* spp. (Chai et al., 2017) and *A. muciniphila* (Liu et al., 2022).

3.3 Colonization resistance and pathogen inhibition

The microbiota prevents pathogens from invading the intestinal ecosystem, a phenomenon known as colonization resistance. The gut microbiota consists of multiple commensal bacteria that may provide colonization resistance through multiple parallel mechanisms, including food struggle, niche exclusion, competitive metabolic interactions, and initiation of host immune response against the harmful bacteria (Pickard et al., 2017). In addition to direct colonization resistance, symbiodinium can modify the intestinal microenvironment to stop the colonization of pathogens. The gut microbiota competed for nutrients, and formed cross-feeding patterns and substrate preferences during evolution to maximize the utilization of existing nutrient. Under steady-state conditions, exogenous strains are unlikely to find an uncompetitive ecological niche and will be forced to compete for nutrients with the normal microbiota in the gut (Sorbara and Pamer, 2019).

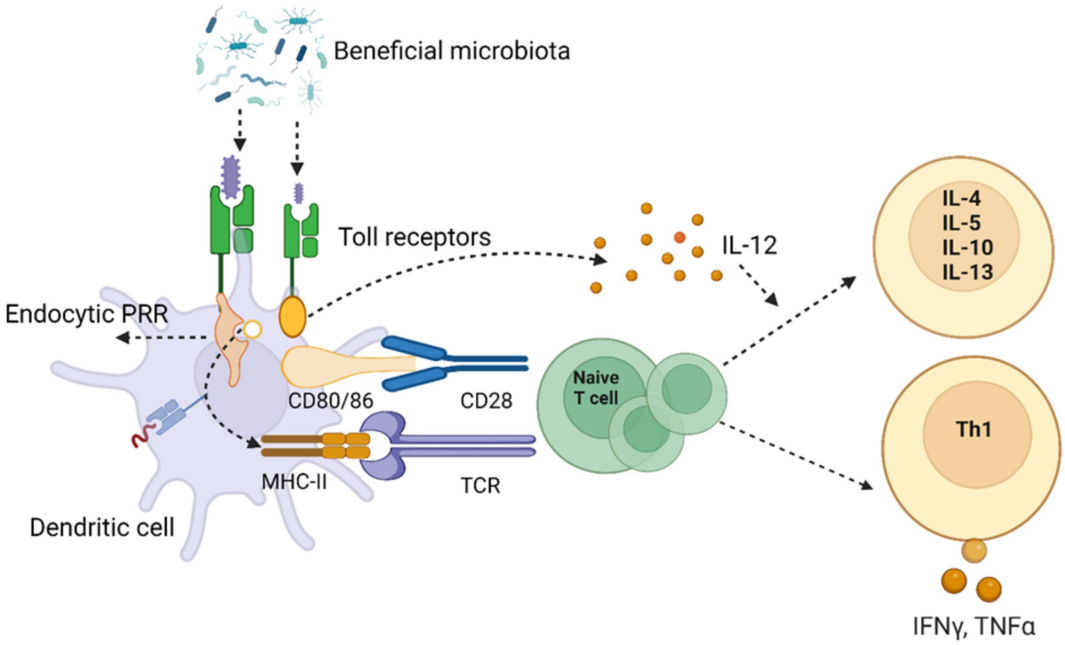

FIGURE 3
The sketch illustrates the intricate interaction between beneficial microbiota and the host immune system through Toll-like receptors (TLRs) on dendritic cells. The binding of microbial components to TLRs triggers a cascade of immune responses. Pattern recognition receptors (PRRs), such as endocytic PRRs, recognize microbial molecules, leading to the activation of dendritic cells. This activation involves upregulation of co-stimulatory molecules like CD80/86, which interact with CD28 on naive T cells through the major histocompatibility complex II (MHC-II) and T cell receptor (TCR) interactions. The dendritic cells release interleukin-12 (IL-12), a key cytokine that promotes the differentiation of naive T cells into T helper cells. Depending on the cytokine environment, T cells differentiate into various subtypes, including Th1 cells, which produce pro-inflammatory cytokines like interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), driving cell-mediated immunity. Alternatively, T cells may differentiate into other subtypes producing anti-inflammatory cytokines such as IL-4, IL-5, IL-10, and IL-13, which modulate immune responses. This mechanism underlines the potential for modulating gut microbiota to influence systemic immunity and the potential therapeutic implications for inflammatory diseases. Created with BioRender.com

Bacteriocins derived from microorganisms have been identified to be active against both Gram-positive and Gram-negative pathogens, typically active against bacteria that are closely related, while others may be more broadly active (Sorbara and Pamer, 2019). There are many kinds of bacteriocins, such as those produced by lactic acid bacteria metabolism can inhibit many bacteria, fungi and viruses, and their antibacterial mechanism of action include destruction of cell membrane, forming transmembrane ion channel and intracellular action of bacteriocins, and interfering with the normal metabolism of bacteria (Pato et al., 2022). The helical structure of Hcp, inner tube and VipA/B outer sheath provides sufficient penetration of the T6SS into the target cell membrane and cell wall (Liang et al., 2018). At present, T6SS is only found in gram-negative bacteria, more than half of the human intestinal Bacteroides genome and more than a quarter of the proteus genome contain T6SS gene, it has a strong bactericidal ability (Coyne et al., 2016). Through direct contact between cells by Type VI Secretion System (T6SS) and physical penetration transport cytotoxic secreted proteins to neighboring cells and eukaryotic cells, can provide colonization resistance to pathogenic bacteria (Burkinshaw et al., 2018). Indirect colonization resistance, SCFAs on the inhibition of bacterial virulence and replication: studies have shown that high concentration of SCFAs inhibit *enterobacteriaceae*, most SCFAs produced in the proximal colon, absorbed by the host to support intestinal epithelial cell metabolism, high concentration of SCFAs lead to intestinal lumen acidification, induced *enterobacteriaceae* bacteria acidification to inhibit its replication mode (Sorbara et al., 2019).

3.4 Intestinal M cells regulation and GIT protection

The gut microbiota is a diverse community of symbiotic bacteria that live in the gastrointestinal tracts of mammals. These bacteria, which are thought to number 40 trillion or more in humans, and the more numbers living in the colon part (Sender et al., 2016). The secretory IgA (SIgA) represent the hallmark of immune response at mucosal sites and contribute to homeostasis via a variety of mechanisms. SIgA antibodies are induced by postnatal exposure to commensal microbiota indicating that these antibodies play a role in sensing commensal microbes and limiting their overgrowth. SIgA antibodies also protect the host by binding to the surface of luminal microbes and toxins to prevent them from attaching to epithelial cells (Boyaka, 2017). IgA binds to the toxin and removes that produced by the harmful microbes, thus keeping the germs out of the intestinal lumen and preserving intestinal homeostasis (Bunker and Bendelac, 2018). The host mucosal immune system has evolved a technique to test the gut microbiota from the intestinal lumen in order to identify these bacteria as toxins. Mucosal tissues are involved in host adaptive immune responses both as effector and inductive sites. Important inductive sites in the intestine are Peyer's patches (PPs) and other gut-associated lymphoid tissue (GALT) (Brandtzaeg et al., 2008; Suzuki et al., 2010). Antigen sampling is the transfer of antigenic material from the external environment across the epithelium to

immune cells located beneath the epithelial layer, is the initial step while starting the antigen-specific mucosal immune responses (Schulz and Pabst, 2013). M "microfold" cells, which are specialized epithelial cells that effectively mediate antigen sampling, are part of the follicle-associated epithelium (FAE) that covers the lymphoid follicles of GALT. The lymphoid follicles of GALT are not reached by antigen or antigen-presenting cells via afferent lymphatics Gebert, 1997. Relatively, M cells offer one of the main routes for directly sampling of commensal enteric bacteria and other antigenic material in the intestinal lumen. Antigens can be quickly transported to dendritic cells that are closely linked to M cells in the subepithelial dome (SED). After processing, antigens are given to T cells, which aid in B-cell maturation, activation and production of IgA-producing cells. Therefore, the secretory IgA response in the intestine is initiated by M-cell-mediated antigen transcytosis (Kraehenbuhl and Neutra, 2000). Thus, the enteric microbiota become a significant element impacting M-cell differentiation. The M cells come into contact with antigens present in the gut lumen. This exposure triggers M cells to capture the antigens, transport them to dendritic cells, and ultimately initiate immune responses (Tahoun et al., 2012).

4 Nutritional contribution of microbiota in mammals

4.1 Feed conversion into nutrients

Together with the diverse microbial ecology, the enzyme activities in the liver and gut mucosa perform a wide variety of metabolic functions and crucial for the host's digestion. As a result, the gut microbiota has a substantial impact on the biochemical composition of the diet and its implications for host health and disease (Rowland et al., 2018). The substrates that are not absorbed or digested in the upper GI tract can be fermented by microbiota in the colon. These substrates consist of foods like proteins, fats, carbohydrates, and other substances that are difficult for the body to digest because of their intricate molecular makeup (Yao et al., 2015). Gut microbes break down complex dietary components such as fibers, starches, and proteins that the host's own enzymes cannot fully process. By fermenting these substrates, they release simpler compounds (e.g., short-chain fatty acids, amino acids, and vitamins). These breakdown products are more easily absorbed by the host's intestinal cells, ensuring efficient nutrient uptake (Rowland et al., 2018). For instance, the rumen, which is regarded as a compartment for anaerobic and methanogenic fermentation, can exploit cellulolytic feeds to increase production, and residing microbiota considerably aiding in feedstuff breakdown (Morgavi et al., 2012). These microorganisms, including the bacteria, archaea, protozoa, and fungi, continuously ferment the food, also break down into constituent parts. These VFAs serve as energy sources for the ruminant, supporting growth, milk production, and overall vitality. Additionally, gut microbes participate in ammonia detoxification, preventing toxic buildup. The most significant pectinolytic species are *Lachnospira multiparus*, *Prevotella ruminicola*, and *Butyrivibrio fibrisolvens*; and these bacteria can break down pectin into oligogalacturonides, leading into significant amounts of acetate, a volatile fatty acid for ruminant metabolism (Duskova and Marounek, 2001).

4.1.1 SCFA and nutrient metabolism to disease prevention

The large intestine microbiota mostly requires nutritional substrates that are not fully digested in the upper digestive tract. When the carbohydrates source becomes scarce, bacteria will seek alternative sources of energy, perhaps leading to the development of more toxic metabolites. However, saccharolytic bacterial fermentation produces metabolites that are generally beneficial (Boyd et al., 2013). The primary byproducts of bacterial fermentation of dietary carbohydrates are gasses and SCFAs. Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, play essential roles in gut health and systemic disease prevention. Butyrate is crucial for maintaining colonocyte energy supply, enhancing gut barrier integrity, and modulating immune responses by promoting the differentiation of regulatory T cells (Tregs) and suppressing inflammation, thus helping to protect against conditions like inflammatory bowel disease (IBD) and colorectal cancer (Liu et al., 2018). Propionate contributes to metabolic health by inhibiting cholesterol synthesis and improving insulin sensitivity, thereby reducing the risk of metabolic disorders such as type 2 diabetes (Zhao et al., 2018). Also, it delivered to the liver, where it helps to gluconeogenesis while also providing energy to epithelial cells. Correspondingly, playing a significant role in satiety signaling due to its interaction with gut receptors, notably G protein-coupled receptors GPR 41 and GPR 43, also known as fatty acid receptors FFAR2 and FFAR3. This interaction may, initiate intestinal IGN (Brown et al., 2003; De Vadder et al., 2014; Karaki et al., 2008). Intestinal gluconeogenesis converts propionate to glucose, which reduces hepatic glucose production, obesity, and thus directly promotes energy homeostasis. Acetate, the most abundant SCFA, has been linked to appetite regulation and blood pressure control, with studies showing its beneficial effects on cardiovascular health (Yang et al., 2022). Acetate, the most common SCFA, is an essential cofactor and metabolite for the growth of other microorganisms. For instance, in the absence of acetate, *Faecalibacterium prausnitzii* requires acetate to grow in pure culture, as it lacks the ability to synthesize this compound independently (Duncan et al., 2004). Acetate is transported to the periphery of the human body, where it is used in lipogenesis and cholesterol metabolism. More recently, studies on mice have demonstrated that acetate is essential for central appetite control (Morgavi et al., 2012). A recent cohort study reported that higher circulating SCFA levels were associated with a 20% reduced risk of type 2 diabetes (Koh et al., 2018), while another study demonstrated that SCFAs reduced the severity of colitis by 30% in experimental models (Furusawa et al., 2013).

4.1.2 Protein and vitamin synthesis

The colonic microbiota is a potent proteolytic agent that can break down ingested dietary protein as well as endogenous protein from host enzymes like mucin, and shed intestinal cells into shorter peptides, amino acids, as well as short and branched-chain fatty acids and gasses like ammonia, H_2, CO_2, and H_2S (Macfarlane et al., 1992). Saccharolysis is predominant in the proximal colon, However, protein fermentation and pH levels rose in the transverse and distal colons. Increased amounts of phenol, indole derivatives resulting from amino acid fermentation, branched-chain fatty acids, and ammonia is linked to protein fermentation (Hylemon et al., 2018; Kalantar-Zadeh et al., 2019). Sulfate-reducing bacteria (SRB), which can convert sulfate compounds to H_2S, produce minor gasses like H_2S and other

sulfur-containing gasses in trace amounts (Mutuyemungu et al., 2023). The amino acids taurine, methionine, and cysteine found in animal proteins, as well as other sulfated polysaccharides like carrageenan, would be the sources of sulfate in the colon (Rey et al., 2013). Research involving aseptic and conventional mice, as well as human volunteers, suggests that the gut microbiota possesses the ability to synthesize certain vitamins. These include vitamin K and various B group vitamins such as biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine (Hill, 1997).

4.2 Meat quality traits

Sustainable meat production is crucial to ensure its availability all across the world and people have access to healthy and high-quality protein. The Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization of the United Nations (FAO) predict that global meat output will grow by 2030 (OECD/FAO 2021). Over the last 10 years, vigorous artificial selection and high energy intake have enhanced daily body weight gain and reduced raising time in many commercial animals, but have accidentally resulted in worse meat quality. Animal gastrointestinal tracts are home for abundant and varied microbial community that is essential to immune system development, meat quality, pathogen elimination, and nutrient digestion and absorption (Chen et al., 2022). The gut microbiota, which is intimately related to host metabolism and health, has been dubbed the second set of the host genome (Noel et al., 2019). Research has indicated a strong correlation between fat metabolism and the gut microbiota (Kuno et al., 2018; Zierer et al., 2018). By sequencing the 16S rRNA gene in the intestinal microbiota of distinct pig gut segments and examining the correlation with meat quality traits (MQTs). Thus, the authors demonstrated that the traits linked to fat deposition in pigs were more significantly influenced by the microbiota of the cecum, colon, and jejunum (Chen et al., 2022). Additionally, a study conducted on castrated Holstein bull as reported by Whon et al. (2021) and examined their gut microbiota profile and MQTs, result suggested an increased extra and intramuscular fat (IMF) storage and a higher relative abundance of the family *Gastrostreptococcus*. Meat quality is complicated term influenced by a variety of elements, most notably customer preferences. Castrated male cattle harbor distinct ileal microbiota dominated by the family *Peptostreptococcaceae* and exhibit distinct serum and muscle amino acid profiles (i.e., highly abundant branched-chain amino acids), with increased extra- and intramuscular fat storage (Whon et al., 2021). According to Zheng et al. (2022), there is a direct relationship between genes associated with muscle metabolism, such as MYLPF, MSTN, ATP2A1, TNNT3, ACTN3, and MYL1, and gut microbial species *B. uniformis*, *B. vulgatus*, *R. inulinivorans*, *C. catus*, *F. prausnitzii*, and *E. rectale*; these species have a direct impact on meat quality. The butyrate-producing bacterium *Faecalibacterium* was linearly connected with the Angus breed, which is known for its high IMF. *Akkermansia*, a mucin-degrading bacterium known for regulating energy expenditure, was found to be more abundant in Brahman calves with lower levels of IMF (Fan et al., 2019). Pigs' carcass configuration and meat quality characteristics were measured in order to estimate the microbiome's heredity. The study revealed a strong positive microbiological correlation between various traits, specifically those associated with meat color and firmness score (Khanal, 2019). Additionally, there were variations in the microbial community's diversity and composition among the various swine breeds. Notably, the Duroc breed, known for its superior meat quality, tenderness, increased flavor, and palatability, had a different microbial community when compared to other breeds (Pajarillo et al., 2014; Pajarillo et al., 2015).

Another, the research demonstrated that the gut flora affects the deposition of intramuscular fat. It is probable that the gut microbiota primarily affects adipose formation through distinct adipogenic pathways (Krause et al., 2020). Furthermore, it was found that fatty and lean-type pigs differed in the abundances of colonic bacteria and bacterial metabolites (Jiang et al., 2016). Similarly, other research revealed a correlation between higher IMF content in pigs and an elevated *Firmicutes* to Bacteroidetes ratio and increased genus Romboutsia abundance in colonic samples (Wu et al., 2021).

4.3 Milk production

Most studies on milk microbiota have primarily focused on mammalian species such as humans (Fitzstevens et al., 2016) as well as domestic animals including cows, goats, sheep, and donkeys (Addis et al., 2016; Falentin et al., 2016). Recent advances in biotechnology enable microbial production of specific Human Milk Oligosaccharides (HMOs), (e.g., 2′-fucosyllactose, lacto-N-neotetraose, 3-fucosyllactose and lacto-N-tetraose). These techniques like whole-cell catalysis and fermentation facilitate efficient biosynthesis of these HMOs (Deng et al., 2020). Microbes also help to synthesize proteins such as caseins and whey protein, and microbial enzymes are involved in lipid metabolism, lactose breakdown, and other processes (Deng et al., 2020). A study found that the efficiency of milk production in cows is linked to their gut microbiome. Less milk-producing cows have undigested nutrients in their large intestine, requiring more beneficial bacteria to breakdown these nutrients, whereas the efficient cows having normal gut microbiota obtained more energy from the undigested nutrients. When Holstein cows eat high-forage diets, their rumen microbiome has more enzymes for breaking down plant components. The high milking cows gut have more fibrolytic bacteria with enzymes, while less producer cows have other class of bacteria associated with lower efficiency (Monteiro et al., 2022).

4.4 Gastric development in weaning mammals

According to this study, dairy calves are born with an underdeveloped GIT and a non-functioning rumen. Compared to adult animal, the rumen has lower proportions and is devoid of some important functional elements, such as the villi in the rumen wall, which are crucial for nutritional absorption (Meale et al., 2017). During the first 3 weeks of life, milk is the primary food source, entering the abomasum through the esophageal groove rather than the rumen. The formation and expansion of the rumen microbiota, particularly starch-degrading bacteria, is triggered by the highly appetizing starting feed which is fermentable into carbohydrates. Increases in microbial biomass and fermentation products alter the rumen's structure and function (Alipour et al.,

2018; Drackley, 2008). Around weaning stage, a fully functional rumen and adult-like microbiota are established (Lallès, 2012). Additionally, in humans, the gut microbiota plays a critical role in the development and differentiation of the intestinal lumen lining epithelial cells as well as the immune system's homeostatic maintenance, which includes tolerance to dietary antigens (Guarner and Malagelada, 2003).

5 The role of microbiota in reproductive health

5.1 Male reproductive efficiency

Since the testis cannot synthesize nutrients, the gut microbiota assists the testis by metabolizing nutrients. The primary modulator of mammalian bone mass is the gut microbiota, which controls the conversion of blood to bone calcium and, consequently, Ca2+ levels in the reproductive system. *Bifidobacteria* and *Lactobacillus* in genetically modified organisms influence the intake of calcium from food. By lowering the pH of the intestine, SCFAs decrease the production of calcium phosphate and increase calcium absorption (D'Amelio and Sassi, 2017). A crucial component of fertilization in mammal is calcium; as it controls sperm motility, which directly affects the likelihood of sperm-egg fusion. The activation of calcium ion channels on the sperm flagellum is essential for facilitating sperm motility into the female reproductive tract, a phenomenon referred to as sperm capacitation (Vyklicka and Lishko, 2020). Folic acid primarily originates from bacterial metabolites and dietary supplements. Proton-coupled folate transporter in colon cells absorbs GTP, erythrose 4-phosphate, and phosphoenolpyruvate to generate tetrahydrofolic acid (THFA), which is then distributed throughout the body via the circulatory system. Genomic analysis has identified various bacteria, including *Salmonella enterica* (Proteobacteria), *Bifidobacterium* spp., (Actinobacteria), *Fusobacterium varium* (Fusobacteria), *Clostridium difficile*, *Lactobacillus plantarum*, *L. reuteri*, *L. delbrueckii* ssp., *Bulgaricus*, and *Streptococcus thermophilus* (Firmicutes), as contributors to THFA synthesis (Yoshii et al., 2019). Intake of folic acid improves semen quality and structural integrity of testicular tissue, especially when animals exposed to reproductive toxins. Folic acid plays a protective role in supporting germ cells against oxidative stress and inflammation, preventing DNA damage and apoptosis. It also protects germ cells from oxidative stress, allowing them to develop and differentiate (Cai et al., 2022). Furthermore, the altered composition of the gut microbiota, including its metabolites, endotoxins, and pro-inflammatory substances, has the potential to affect gut permeability and immune function, can adversely affect the reproductive system and the immune environment of the testis (Guo et al., 2020). The gut microbiome, considered an endocrine organ, impacts the reproductive endocrine system through sex hormone fluctuations (Ashonibare et al., 2024). The amount of testosterone in the blood can also be altered by the gut microbiome (Qi et al., 2021). The gut microbiota has been identified as a key regulator of androgen production and metabolism. By producing enzymes, the gut microbiota can generate and convert androgens, actively partaking in microbial processes that break down testosterone (Lv et al., 2024). For example, *Clostridium scindens* exhibits a high potential for converting glucocorticoids into androgens, while certain proteobacteria possess the ability to degrade androgen. These intricate interactions between gut microbes and androgen metabolism significantly update our understanding of male reproduction (Emenike et al., 2023; Wang et al., 2014; Yang et al., 2016).

5.2 Female reproductive efficiency

The female reproductive tract is home to a diverse ecosystem of chemicals, immune components, host cells, and microbes. The complex interactions that occur among bacteria, immune cells, and host cells within the female reproductive system help to maintain reproductive tract homeostasis (Gholiof et al., 2022). The gut microbiome, which is considered an extended endocrine organ, plays an important role in female reproductive health (Chadchan et al., 2022). According to microbiome's evaluations, the vaginal microbiota accounts for around 9% of the overall human microbiome (Saraf et al., 2021). The bacterial genera like *Prevotella*, *Bifidobacterium*, *Gardnerella*, *Atopobium*, *Megasphaera*, *Sneathia*, and *Anaerococcus* are associated with various reproductive stages, including gamete development, fertilization, the initiation and preservation of pregnancy, and the microbial colonization of the developing fetus or infant (D'Argenio, 2018; Franasiak and Scott, 2015; Moreno and Simon, 2018).The secretion of β-glucuronidase can be modulated by the gut microbiota, which is crucial and impact the estrogen levels. Dysbiosis or decrease in the diversity of the gut microbiota can cause fluctuations the estrogen levels in blood and β-glucuronidase activity. These variations can contribute to obesity, metabolic syndrome, cancer, endometrial hyperplasia, endometriosis, PCOS, and infertility (Baker et al., 2017; Chadchan et al., 2022). As demonstrated in Table 2, the composition of gut microbiota varies significantly across different mammalian species, with notable differences in key bacterial phyla that have been linked to reproductive health outcomes.

5.3 Mother-newborn bond

The mother's oral, stomach, and vaginal microbiota are fluctuating throughout pregnancy. These changes are caused by a variety of factors, including host genes, antibiotic use, infections, diet, and stress (Codagnone et al., 2019; Goodrich et al., 2016; Kim et al., 2017; Zhou et al., 2020). According to Romero et al.'s (2014) study, a large number of *Lactobacillus* spp., were discovered among other vaginal microbiota in healthy pregnant women, and this species is more stable in these women than in nonpregnant healthy women. About 90% of *bifidobacteria* and were found in the microbiota of breastfed infants, whereas 40–60% were found in formula-fed infants. Furthermore, breastfed newborns develop their gut microbiota more quickly than infants nourished via formula milk. Furthermore, studies indicated microbial variations between breastfed and non-breastfed infants, with *Bifidobacterium adolescentis* colonization being more common in the breastfed newborn and *Bifidobacterium catenulatum* lacking (Coppa et al., 2011).

TABLE 2 A comparative analysis of the microbiota across diverse mammalian species and its influence on reproductive processes.

Aspect	Human	Non-human	Rate	Cattle, Sheep	Horse
Vaginal microbiota	*Lactobacillus* species dominate, with acidic pH aiding infection defense, while disruptions like bacterial vaginosis affect fertility (Olson et al., 2018).	A more diverse microbiota, less dependent on Lactobacillus, relies on immune adaptations for infection defense (Nuriel-Ohayon et al., 2016).	The vaginal microbiome shifts significantly during the estrous cycle, with reduced Lactobacillus dominance and a greater influence on mating behaviors (Miller et al., 2016).	*Lactobacillus* is less prevalent, with microbial shifts influenced by reproductive cycles. Infections like metritis reduce fertility (Santos and Bicalho, 2012).	In marsupials, pouch microbiota varies with reproductive architecture, while in horses, vaginal microbiome diversity impacts fertility (Chhour et al., 2010).
Seminal microbiota	A diverse microbiome influences sperm motility, with an overgrowth of bacteria like Enterococcus associated with male infertility (Jendraszak et al., 2024).	The seminal microbiome in non-human primates affects sperm quality, but it is less studied than in humans (Camargo et al., 2017).	Microbial imbalances in seminal fluid are less studied but can similarly affect sperm motility and reproductive success, as in humans (Bicalho et al., 2017).	The seminal microbiome influences sperm quality in animals, with homogeneous compositions linked to higher fertility (Castillo et al., 2015).	Microbial imbalances in horse seminal fluid can impair sperm motility and fertility, despite a diverse seminal microbiota composition (Al-Essawe et al., 2018).
Microbial changes during pregnancy	As gastrointestinal diversity decreases, *Lactobacillus* dominance in the vaginal microbiome rises. Dysbiosis may lead to preterm birth and preeclampsia (Koren, Goodrich, Cullender, Spor, Laitinen, Bäckhed, et al., 2012).	The vaginal microbiota in pregnancy changes more subtly than in humans, relying on immune system regulation (Weichhart et al., 2015).	Gut and vaginal microbiota shifts during pregnancy facilitate microbial transfer to the child, influencing immune system development (Rautava et al., 2012).	Pregnancy has a smaller impact on livestock microbiota, but reproductive diseases like metritis can be detrimental (Liu et al., 2022).	Marsupials experience unique microbial changes due to the pouch environment, while in horses, microbial stability during pregnancy is crucial for fetal health (Hand et al., 2016).
Microbial transfer to offspring	Vaginal birth introduces beneficial bacteria to newborns, and breastfeeding offers additional microbial exposure, crucial for immune development (Dominguez-Bello et al., 2010).	Similar to humans, though with different bacterial species and less *Lactobacillus* dominance, breastfeeding still transfers beneficial bacteria (Łubiech and Twarużek, 2020).	Vaginal delivery and breastfeeding support early microbial colonization, aiding the development of the newborn's immune system (Bäckhed et al., 2015a).	Vaginal delivery and colostrum transfer crucial microorganisms for infant survival, while microbial diversity supports immune priming (Reynolds and Bettini, 2023).	In marsupials, exposure to pouch microbiota is vital for offspring survival, while in horses, similar microbial transfer occurs during birth and nursing (Zhong and Zhong, 2016).
Reproductive cycle and microbial shifts	The microbiota remains largely stable throughout the reproductive cycle, except for pregnancy-related changes that protect the fetus and support reproductive health (Borody and Khoruts, 2011).	Hormonal changes during the reproductive cycle significantly alter microbial composition, directly affecting reproductive success (Antwis et al., 2019).	Microbial composition shifts with the estrous cycle, affecting reproductive behaviors and outcomes (Qi et al., 2021a).	Microbial shifts during the menstrual cycle enhance fertility and help prevent diseases like metritis and vaginitis (Molina et al., 2020).	Microbial changes in seasonal breeders like horses align with hormonal shifts, boosting reproductive success and supporting pregnancy (Yatsunenko et al., 2012).
Impact of dysbiosis on reproduction	Dysbiosis is linked to infertility, premature birth, and bacterial vaginosis. In men, seminal microbiota imbalances reduce sperm motility (Baker et al., 2018).	Dysbiosis leads to reproductive disorders like infertility, though research in this area is less advanced compared to human studies (Markle et al., 2013).	Dysbiosis affects fertility and pregnancy outcomes by disrupting reproductive health and immune system regulation (Morgan, 2015).	Dysbiosis leads to reproductive diseases like metritis, mastitis, and vaginitis, significantly lowering reproductive success (Bicalho and Oikonomou, 2013).	Dysbiosis in marsupials can disrupt pouch microbiota, while microbial imbalances in horses are linked to reduced fertility and reproductive issues (Garcia-Garcia et al., 2022).

6 Biological association and implications for human and veterinary medicine

6.1 Gut-brain axis

Gut microbes actively contribute to neurodevelopmental processes, including the formation of the blood–brain barrier, neurogenesis, microglial maturation, and myelination (Cerdó et al., 2020; Parker et al., 2020). The gut-brain axis is a bidirectional communication network connecting the intestinal and central nervous systems. This network extends beyond anatomical connections to include endocrine, humoral, metabolic, and immunological pathways. The autonomic nervous system, hypothalamic–pituitary–adrenal (HPA) axis, and gastrointestinal (GI) nerves form key links between the gut and brain, enabling the brain to regulate intestinal functions, such as immune cell activity, while allowing the gut to impact mood, cognition, and mental health. Ongoing research is uncovering the mechanisms by which microbiota influence the brain's emotional and cognitive centers, both directly and indirectly. Studies have demonstrated that fluctuations in microbiota are associated with alterations in these communication systems (Mayer et al., 2014). Additionally, research suggests that the composition of the gut microbiota may play a role in influencing fetal and neonatal brain development (Douglas-Escobar et al., 2013). Bacterial metabolites, particularly short-chain fatty acids (SCFAs) produced by the fermentation of dietary carbohydrates, act as key humoral modulators. These microbiota-derived SCFAs can cross the blood–brain barrier and have been shown to regulate microglial homeostasis, which is crucial for proper brain development, tissue maintenance, and behavioral modulation (Mayer et al., 2015). SCFAs regulate the secretion of gut peptides from enteroendocrine cells and influence the production of gut-derived serotonin by enterochromaffin cells. Both processes play a crucial role in modulating gut-brain hormonal communication (Wang and Kasper, 2014). The gut produces approximately 95% of the body's total serotonin, with most of it found in plasma. While serotonin has key roles in gut function and peripheral metabolism, it can also locally activate afferent nerve terminals that connect directly to the central nervous system (Macfabe, 2013). Treatment with *Lactobacillus rhamnosus* reduced stress-induced corticosterone levels and alleviated anxiety- and depression-related behaviors in rats. Notably, in mice that underwent vagal nerve dissection, no neurochemical or behavioral changes were observed, confirming that the vagus nerve is the primary pathway for communication between gut bacteria and the brain (Kim and Shim, 2023). A balanced gut microbiota plays a crucial role, both directly and indirectly, in maintaining the environment necessary for optimal neural development (Sarubbo et al., 2022). A recent comparative study of germ-free (GF) and specific pathogen-free (SPF) mice has identified several gut microbial compounds that can cross the placenta and enter the fetal compartment, where they influence and regulate prenatal developmental processes (Pessa-Morikawa et al., 2022). Tail biting is a prevalent and harmful issue in intensive pig farming. A recent study estimated that tail biting in pigs could reduce net profit by up to USD 23.00 per pig, leading to annual losses amounting to millions of dollars for the pork industry (Henry et al., 2021). Regarding tail biting and the porcine microbiome, no significant difference in alpha diversity was observed between tail-biters and the control group. However, a consistent difference in beta diversity was noted among tail-biters, victims, and the control groups (Verbeek et al., 2021). In pigs exhibiting tail-biting behavior, victims of tail-biting, and those showing other anxiety-related behaviors, certain Firmicutes families and orders, specifically Clostridiales (including *Ruminococcocus*, Lachnospiraceae, and Clostridiales Family XII), showed a relative increase in abundance. In contrast, other Firmicutes, particularly Lactobacillus spp., exhibited a relative decrease in abundance. The composition and abundance of gut microbiota, particularly Firmicutes and Bacteroidetes, have been linked to various mental disorders in humans, including anxiety, depression, bipolar disorder, autism spectrum disorder (ASD), and schizophrenia (Xiong et al., 2023).

6.2 Biological association between microbiota and host

Numerous physiological function of host, such as nutritional and metabolic processes (Degnan et al., 2014), immune system modulation and regulation (Francino, 2014), and brain and behavior development (Hsiao et al., 2013), are associated with the microbiota. According to recent research, the host and normal microbiota interact in the following four ways: firstly, the microbiota acts as a barrier against pathogens; secondly, it modifies the host mucosa's permeability; thirdly, it influences the host's capability to extract energy from food and use it metabolically; and lastly, it influences the immune system. By these four ways of interaction also increase the host's susceptibility to diseases when the normal microbiota is altered. For instance, commensal bacteria exist in the outer mucus layer epithelial tissues, therefore changes in the normal microbiota affect intestinal mucosa permeability. When the body is healthy, the inner mucus coating works as a physical barrier, preventing bacteria from making direct contact with the epithelial layer (Hertli and Zimmermann, 2022). Intestinal permeability dysfunction can result from disruption of mucosal development, such as through dysbiosis, and has been linked to an inclination toward immunological disorders (Groschwitz and Hogan, 2009). Metabolites from various commensal bacteria, such as *Bifidobacterium lactis*, *Bacteroides fragilis* and *Akkermansia muciniphila* (Lindfors et al., 2008; Hsiao et al., 2013; Chelakkot et al., 2018; Everard et al., 2013; Plovier et al., 2016), affect mucin production and keep tight junction appearance to maintain the intestinal barrier.

Regarding the third point, the gut microbiota has determined the bioavailability of vitamins derived from food. A good example is the conversion of vitamin K1 to vitamin K2, which is assisted by commensal bacteria such as *Veillonella*, *Eubacterium lentum*, *Enterobacter*, and *Bacteroides* (Biesalski, 2016; Blacher et al., 2017). Vitamin K acts as a cofactor to regulate the coagulation cascade and immunological processes in mammalian species. The presence of K2 may lower the incidence of osteoporosis and coronary heart disease (Beulens et al., 2013). The body absorbs tryptophan (Trp), an essential amino acid, through food. The intestinal microbiota has the ability to metabolize it and produce aryl hydrocarbon receptor (AhR) ligands. The body's various cells express AhR, which affects a number of host immunological responses and pathways, such as the cell cycle, immune system, neurological signaling, and reactions to xenobiotics, antioxidants, and hormone-like estrogen (Agus et al., 2018). AhR ligands regulate the growth activity, and synthesis of metabolites in addition to mucosal immune cells. Tryptophanase is a bacterial enzyme and the following group of *Lactobacilli* (Lamas et al., 2018), *Peptostreptococcus russellii* (Wlodarska et al., 2017), and *E. coli* (Agus

et al., 2018; Alexeev et al., 2018) use it for converting tryptophan into indole. Whereas, indole stimulates the synthesis of IL-22, which improves intestinal homeostasis (Agus et al., 2018; Alexeev et al., 2018; Lamas et al., 2018). Multiple cytokines influence intestinal epithelial cells, increasing proliferation and the generation of antimicrobial peptides (Lamas et al., 2018). Furthermore, AhR ligands reduce the possibility of intestinal pathogen colonization. *In-vitro* experiment showed that indole-3-acetonitrile prevents the *Candida albicans* multiplication in the growing biofilms and adhering to gut epithelial cells (Oh et al., 2014). The lower production of these ligands by the microbiota in IBD patients may be linked to the decreased production of AhRs by the immune cells like (CD3+, CD4+, CD56+, and CD25+) (Lamas et al., 2018). Moreover, interferon (IFN)-γ secretion is decreased and IL-22 production is increased by AhR ligands, which counteract inflammatory responses (Monteleone et al., 2011). In the future, therapies for autoimmune disorders, infections, and chronic inflammation may be developed by focusing on the IL-22 pathway. *Bifidobacterium infantis* activates the rate-limiting enzyme like indoleamine 2,3-dioxygenase-1 (IDO-1) for the conversion of Trp to kynurenic acid. This molecule is crucial for both immunological responses and neuronal processes. Experiments in rats have shown that *Bifidobacterium infantis* may have an antidepressant effect (Tian et al., 2019). Furthermore, the microbiota can convert Trp into indole, promoting colonic L cells to generate GLP-1 and perhaps contributing to the genesis of metabolic syndrome (Chimerel et al., 2014). Tryptophan hydroxylase is an enzyme that converts Trp to tryptamine, and it is activated by *Lactobacillus bulgaricus*, *Clostridium sporogenes*, and *Ruminococcus gnavus* (Williams et al., 2014). Tryptamine increases the inhibitory response of cells to serotonin by binding to trace amine-associated receptors in the brain (Williams et al., 2014). Tryptamine also attaches itself to the sigma-2 receptor in mice, which may play a role in the onset of Alzheimer's and cancer (Williams et al., 2014; Yang et al., 2020). Tryptamine causes enterochromaffin cells in the gastrointestinal tract to secrete more serotonin. Variations in intestinal serotonin levels are thought to affect intestinal motility and may contribute to the pathophysiology of inflammatory bowel disease (Williams et al., 2014). Tempering serotonin receptors may aid in the treatment of irritable bowel syndrome (IBS) because serotonin is an important neurotransmitter for signaling in the enteric nervous system (Williams et al., 2014). Carbohydrate metabolism pathways are investigated to determine the fundamental processes of host-microbiome metabolic interactions. Microbes undergo fermentation, a metabolic process that converts sugars into several byproducts, including butyrate, propionate, and acetate, which are classified as short-chain fatty acids (SCFAs) (Jansma and El Aidy, 2021). SCFAs, in particular, have a significant impact on host metabolism, immunological function by providing energy to gut epithelial cells and to beneficial bacteria, gut barrier function, gut cell proliferation, and even gut-brain axis communication (Shtossel et al., 2024). The primary ligand for GPR41 (Free Fatty Acid Receptor 3, or FFAR3), binds to propionate and activates GPR41. Propionate-induced GPR41 activation can control a number of cellular reactions, including the release of hormones like peptide YY (PYY), which lowers gut motility and increases energy expenditure. GPR43 (Free Fatty Acid Receptor 2, FFAR2) ligands such as acetate and propionate can cause the release of hormones like glucagon-like peptide-1 (GLP-1), which can have an impact on insulin secretion and glucose homeostasis while the intestinal gluconeogenesis (IGN), butyrate has beneficial effects on glucose and energy homeostasis. This complex interplay emphasizes how crucial the gut microbiota and its metabolites are to preserving a healthy and mutually beneficial relationship with the host and shown in (Figure 4).

6.3 Biological association between pathogen and host microbiota

Animals live in symbiosis with numerous microbial species and it is widely recognized that host microbiota help to prevent or fight infection (Britton and Young, 2014; Chiu et al., 2017; Lamousé-Smith et al., 2021; Shanahan, 2010), as like changes in the microbial landscape, as well as microbiota components, have the potential to exacerbate infections and disease severity. Pathogens and pathobionts can cause opportunistic infection by exploiting microbiota metabolites or taking advantage of a host's defenses being depleted and changing environment. The microbiota may potentially support a more virulent evolutionary pathway for invading diseases (Stevens et al., 2021). It has shown that the emergence of pathogenic bacterial species and disruption of the gut microbial community, known as dysbiosis, are linked to the development of a number of systemic illnesses, including autoimmune diseases (Mousa et al., 2022). The host microbiota serves as a pathogen barrier or deterrent by acting through innate immune hubs, has the ability to prevent or alleviate an increase in inflammation. Various components, such as epitheloid cells' microbiota, innate lymphocytes (Han et al., 2013), and adaptive lymphocytes (Kubinak et al., 2015), all interact with pattern recognition receptors. Noteworthy examples include the modulation of goblet cell functions (Wang et al., 2015) and the control of granulopoiesis through Myd88/TICAM by microbiota-released substances (Balmer et al., 2014). Related to changes the microbial landscape, intestinal microbiota barrier function is exemplified by *Clostridium difficile* infection (CDI). When a healthy microbiota is lost, conditions arise that facilitate the infection and disease-causing potential of *C. difficile*. Antibiotic-induced dysbiosis (abnormal microbiota) and subsequent *Clostridioides difficile* infection (CDI) have been linked to alterations in epithelial permeability, TH17 function, and TLR signaling. This strong evidence from both human and veterinary studies demonstrates that restoring the microbiota through fecal microbiota transplantation (FMT) resolves Clostridioides difficile infection (CDI) and increases resistance to recurrence, lending credence to the theory that changes in the microbiota cause CDI. In these studies, fecal transplants were administered either orally or rectally, transferring healthy donor microbiota into the gastrointestinal tract of affected animals, effectively re-establishing microbial balance and suppressing pathogenic bacteria (Barbara et al., 2021).This approach has shown promise in veterinary medicine, particularly in cases of chronic gastrointestinal diseases.

Microbiota metabolites have a diverse impact in host health, including priming the immune structure, acting as antimicrobials, and aiding host metabolism (McCarville et al., 2020; Rooks and Garrett, 2016). However, the same metabolites may also serve as a food source for occupying pathogens. Metabolic interactions create new environments that could potentially support pathogens, boosting their ability to produce energy and become more virulent (San Roman and Wagner, 2018). For instance, the gut bacteria *Bacteroides thetaiotaomicron* can worsen infections caused by enterohaemorrhagic *Escherichia coli* through metabolic interactions (Curtis et al., 2014).

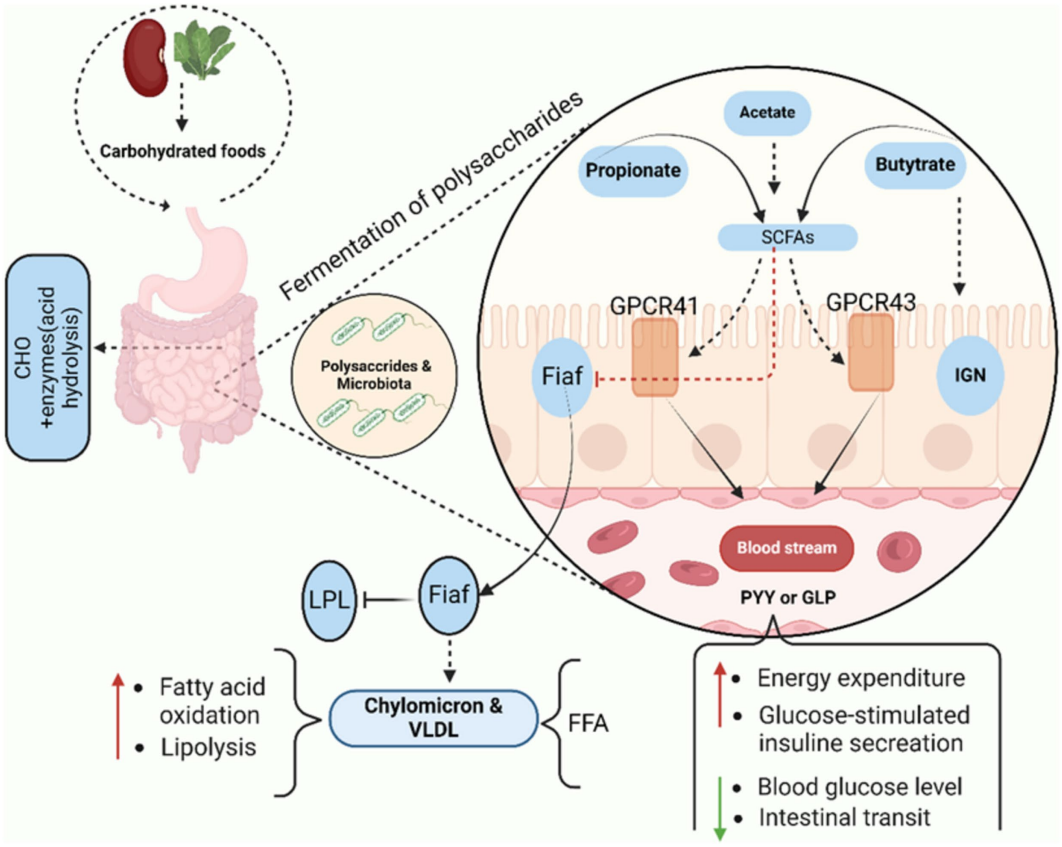

FIGURE 4
This pathway explores the mechanisms underlying metabolic interactions between host and microbiota. Fermentation of polysaccharides where microorganisms convert these sugars into various byproducts, including short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate. Propionate (Free Fatty Acid Receptor 3, FFAR3) is a primary ligand for GPR41 and activates GPR41. The GPR41 can influence a variety of cellular responses, including hormone release like peptide YY, and increase energy expenditure while decreasing gastrointestinal motility. Acetate and propionate are ligands for GPR43 (Free Fatty Acid Receptor 2, FFAR2) and lead to the release of hormones such as glucagon-like peptide-1 (GLP-1), affecting insulin secretion and glucose homeostasis. Butyrate works on Intestinal gluconeogenesis (IGN) thus regulate energy homeostasis. Fiaf (Fasting-Induced Adipose Factor) which inhibits lipoprotein lipase (LPL), and releases fatty acids from circulating chylomicrons and VLDL lead to promotes fatty acid oxidation and lipolysis (breakdown of stored fat). SCFAs act as metabolic signals that influence various pathways, including fat metabolism, insulin secretion, and gut motility. Also, this interaction offers insights into therapeutic strategies for metabolic disorders such as obesity, diabetes, and dyslipidemia, by modulating gut microbiota or SCFA production. Created with BioRender.com.

Unique microbial populations influence the outcomes of infections by producing different metabolites. The intricate nature of these interactions poses a challenge in microbiome research.

7 The modeling approaches used to study the microbiota

7.1 Germ-free animals as crucial models for investigating host-microbial interactions

Valid experimental models for examining the host-microbial interactions in health, disorders, and diseases are germ-free (GF) animals (Al-Asmakh and Zadjali, 2015; Bhattarai and Kashyap, 2016). While mice are commonly employed in germ-free models, other species, such as zebrafish, may also be utilized in these investigations (Melancon et al., 2017). For a variety of research projects, germ-free pigs, chickens, and dogs were also raised (Harding et al., 2010). Numerous fields, including cancer therapy, metabolism, diabetes, reproduction, cardiovascular issues, and bone homeostasis, can benefit from its application. Recently, the significance of the gut-brain axis in the brain development of mammals has been established (Diaz Heijtz et al., 2011). Mice, in particular, have been used in a number of germ-free animal studies on behavioral and brain disorders or diseases, including anxiety, depression, schizophrenia, and autism (Horne and Foster, 2018; Neufeld et al., 2010). Consequently, GF animals are an appropriate and accessible model for research on the gut-brain axis. It is feasible to develop genetically modified mice as GF to investigate the interactions between specific genes and the gut microbiota. The phylogenetic composition of the human microbiota can be recapitalized through the inoculation of human gut microbiota into humanized gnotobiotic GF mice. These models serve as effective tools for better understanding the composition of gut microbiota in systems that resemble humans (Al-Asmakh and Zadjali, 2015). Mice of various breeds can also be utilized as a germ-free model. For instance, GF models have been utilized to study anxiety and type 2 diabetes in Swiss-Webster and C57BL/6 mice, respectively (Hansen et al., 2014). In animals given antibiotics, phenotypic transfers through microbial transplantation are also feasible. The most reliable controlled models for microbial transplantation appear to be GF animals (Croswell et al., 2009).

GF animals are used as models in preclinical research to better understand the impact of bacteria on host development and

function, rather than to replicate human conditions. Furthermore, we were able to ascertain the influence of a particular strain of bacteria on various health-related problems by colonizing the animals with either a single strain or a combination of known strains. There are a few substitutes for the germ-free murine model. Numerous approaches have been investigated as substitutes for the germ-free (GF) murine model, including probiotic diets, fecal transplants, antibiotic therapy, and humanization of mice by colonizing them with human microbiota. These methods aim to manipulate the gut microbiota and study its effects on host physiology. Fecal microbiota transplants (FMT) involve transferring microbiota from healthy donors into germ-free or antibiotic-treated mice, while humanized mouse models are generated by colonizing GF mice with human fecal material to mimic the human microbiome. Although challenges exist in extrapolating results from mouse models to humans due to differences in microbiota composition, GF animals remain the most effective alternative to date for studying microbiota-related health outcomes, particularly in the context of host-microbiota interactions (Gabay et al., 2020).

7.2 Interactions through mono and bi-associated gnotobiology models

The study of particular microbial species or strains that colonize in GF animals is known as gnotobiology. Research involving animals with a single or pair of commensal species allows us to study how microbes affect their hosts in a simple ecosystem. Mono-associated animal models can help us understand the microbe's niche, resources, and host response. Likewise, studies on bi-associated animals can shed light on the interactions that occur between a pair of microbes and their host, as well as whether the competition that occurs during colonization for resources and space which affects the functional roles that the microbes establish within the gut ecosystem (Bäckhed et al., 2005; Ley et al., 2008; Sadeghi et al., 2023). The fecal models of *Bacteroides thetaiotaomicron* and *Eubacterium rectale* developed by Mahowald (Mahowald et al., 2009) demonstrated that both organisms undergo a significant shift in gene expression patterns when transitioning from mono-association (where each organism was introduced individually into germ-free mice) to co-colonization (where both organisms were introduced together). This shift was identified through comparative transcriptomic analyses, which revealed how these bacteria respond differently to their environment when colonizing the gut in the presence of other microbial species. The study highlights the complex interactions between gut microbes and their adaptive behavior within microbial communities. This is because the variation in the gut microbiome is determined by the composition of its members and the sequence in which they colonize each other. These models are useful for understanding the specific niches occupied by individual members of the gut microbiota. However, they are further complicated by the fact that these bacteria respond differently to co-colonization based on species and sequence. For example, in newborn human babies, the obligate anaerobes take over the GIT after the facultative anaerobes (Mahowald et al., 2009). Once we start introducing GF animals to the world, it's not always clear how their interactions with different commensal microbes will play out. It's not always easy to predict how they'll behave in a natural state.

7.3 The altered schaedler flora: a consistent model for investigating gut ecosystems

Russell W. Schaedler introduced the idea of multiple-associated animal models in the middle of the 1960s (Dewhirst et al., 1999). The aim was to create a standardized gut microbiota to serve as a reliable research tool. This was accomplished by intentionally populating GF mice with eight specific bacterial strains derived from conventional mice. The widely utilized standardized poly-associated flora in current laboratory settings is the Altered Schaedler Flora (ASF), a modification of the original Schaedler Flora dating back to 1978. The ASF, which includes four fusiform bacteria that are highly sensitive to oxygen (EOS), two *Lactobacillus* spp., a cousin of *Bacteroides distasonis*, and a spiral-shaped bacterium from the Flexistripes phylum, was recently characterized using 16S rRNA profiling (Dewhirst et al., 1999). It is increasingly important for this set of standardized flora to accurately mimic the ecosystem of gut of a conventional animal, rather than focusing solely on the specific identities of the bacterial strains constituting the ASF. Fifty percent of the bacterial strains present in the ASF consist of *Firmicutes* EOS bacteria. Multiple recent studies examining the gut microenvironment consistently reveal that EOS bacteria make up the predominant portion of the gastrointestinal microbiota in mice and rats, surpassing aerobes by a ratio of at least 1,000:1 (Savage, 1970), and facultative anaerobes by a ratio of 100:1. Two of the ASF's members are Lactobacillus spp., an aerotolerant subgroup of Firmicutes that frequently colonize human stomachs and small intestines as well as other conventional vertebrate mammals (Roach et al., 1977). Because ASF animal models are colonized with a specific set of intestinal flora, they are thought to be beneficial. Animal shelters differ significantly in terms of their flora, as do cages within the same facility. Therefore, by reducing variability arising from variations in the gut ecosystems of different research animals, the colonization of animals with the standardized ASF flora enhances cross-study comparability. ASF has been successfully introduced into new mouse strains as knockout strains and transgenics through embryo transfer. According to recent 16S rRNA sequencing of the mouse gut flora, the ASF in a mouse colony is stable over an extended period of time (i.e., free of contaminating bacteria) (Stehr et al., 2009). These stability tests do not, however, remove the necessity of actively monitoring the ASF's composition, as unnoticed changes in the flora may materially impact the results of later experiments. Using a consistent gut flora in ASF animal models is beneficial. However, the intricacy of the typical intestinal microbiota, which includes around 800 to 1,000 bacterial species, cannot be accurately mimicked by an intestinal microbiota consisting of only eight bacterial species (Bäckhed et al., 2005). The ASF can accurately represent the dominant phyla found in a conventional vertebrate animal, but it is not expected to show host-microbiome interactions that are entirely comparable to those found in a natural gut ecosystem because so few organisms can replicate its community dynamics.

8 Conclusion and future implications for practice

In conclusion, we investigate the unique microbiota of the oral, respiratory, skin, gut, and genital tracts, emphasizing their individual

roles and cumulative impact on mammalian host health. Also, highlights the pivotal role of gut microbiota in regulating health, production, and reproduction in both humans and animals, influencing key metabolic, immune, and reproductive processes. Understanding these intricate host-microbiota interactions has profound implications across life cycle, host signaling pathways. Finally, authors investigate into modeling approaches for microbiota research, including germ-free animal models, mono-associated and bi-associated models, and poly-associated animal models. These models provide essential tools for studying the dynamic nature of microbial communities and their effects on host organisms. Translationally, the findings suggest that microbiota-based therapies hold promise for enhancing overall health and productivity in agricultural species, as well as improving reproductive health in clinical applications. Despite significant progress in understanding gut microbiota's role in mammalian health, production, and reproduction, several gaps remain. Causality between specific microbial communities and host outcomes is difficult to establish, with most studies relying on correlative data. Research has primarily focused on model organisms, limiting broader application to diverse species. Environmental, dietary, and geographic variations also complicate universal conclusions. Additionally, most studies examine short-term effects, underscoring the need for more longitudinal research to understand the sustained impact of microbiota on host physiology. Addressing these gaps will enhance the development of microbiota-based interventions.

Implications for practice

- Livestock management

Modulating the gut microbiota through the use of prebiotics, probiotics, or optimized feed strategies can enhance digestion and nutrient absorption. This approach may lead to improved feed efficiency, reduced feed costs, and accelerated growth rates.

Maintaining a balanced gut microbiota plays a crucial role in enhancing immune function and reducing the risk of disease. Probiotic supplements and fecal microbiota transplantation (FMT) present promising alternatives to antibiotics for disease prevention.

Farmers can reduce their reliance on antibiotics by promoting a healthy microbiome in livestock, which helps mitigate the risk of antibiotic resistance in animals and the human food chain.

Animals with a balanced microbiota may produce higher-quality meat and dairy products, potentially enhancing taste, texture, and nutritional value.

- Veterinary practices

Veterinarians can design more precise and individualized treatments for animals by analyzing the composition of their microbiome. This tailored approach may enhance therapeutic outcomes for diseases associated with microbial dysbiosis, such as gastrointestinal disorders, dermatological infections, and respiratory conditions.

The analysis of microbiota composition can facilitate early diagnosis of certain diseases, enabling more effective and timely interventions. This approach is particularly advantageous for detecting subclinical infections and identifying animals at risk for metabolic or inflammatory conditions.

Probiotics, synbiotics, and fecal microbiota transplantation (FMT) can be integrated into routine veterinary care, offering less invasive alternatives for managing chronic conditions. These approaches may reduce the reliance on pharmaceutical medications while promoting long-term health in animals.

- Public health

Regulating cattle microbiota can help reduce the prevalence of harmful pathogens, such as Salmonella, Campylobacter, and *E. coli*, in the food supply, thereby decreasing the risk of foodborne illnesses.

Reducing antibiotic use in livestock through microbiota management directly addresses the escalating public health threat of antibiotic-resistant microorganisms. Livestock serve as a significant reservoir for resistant pathogens, which can be transmitted to humans through food, water, or direct contact.

A deeper understanding of animal microbiota can contribute to the prevention of zoonotic diseases, such as avian influenza and coronaviruses, by identifying and managing microbial factors that facilitate pathogen transmission between animals and humans.

Microbiota regulation in cattle may help reduce the environmental impact of livestock farming. Animals with a healthier and more balanced gut microbiota tend to produce lower methane emissions, a potent greenhouse gas, and require fewer resources for feeding and maintenance.

Author contributions

IK: Writing – review & editing, Writing – original draft, Conceptualization. NN: Writing – original draft, Investigation, Writing – review & editing. HC: Writing – review & editing, Investigation, Funding acquisition. SK: Writing – review & editing, Validation, Investigation. MC: Writing – review & editing, Visualization. ZW: Writing – review & editing, Visualization, Supervision. XX: Writing – review & editing, Funding acquisition.

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Conflict of interest

MC was employed by Fisugarpeptide Biology Engineering Co. Ltd.

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Publisher's note

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