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Doctoral Dissertation

Characterization of Bacterial Communities Associated with Rootless Duckweed (*Wolffia globosa*) and Their Effects on the Host Biomass Productivity

根を持たないウキクサ（*Wolffia globosa*）と共棲する細菌群集の特徴付けとそれらがバイオマス生産に及ぼす影響

SAIMEE YUPARAT

(サイミー ユパラット)

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Division of Sustainable Energy and Environmental Engineering

Graduate School of Engineering

The University of Osaka

Abstract of Dissertation

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Name: SAIMEE YUPARAT

Supervisor: Professor IKE MICHIHIKO

Duckweed has attracted increasing attention as a promising bioresource because of its rapid growth, global distribution, and nutritionally valuable biomass. Increasing evidence indicates that their growth and physiology are strongly influenced by the associated microbiomes and specific duckweed-associated bacteria (DABs). However, most previous work has focused on rooted duckweeds such as *Lemna* and *Spirodela*, the microbiomes of *Wolffia*, which holds an exceptionally promising bioresource and for which the establishment of efficient cultivation technologies is desired, remain insufficiently characterized. Given its unique morphology, the structure and function of the *Wolffia* microbiome likely differ from those of rooted duckweeds. Thus, clarifying the microbiome of *Wolffia* is essential for providing insight to enhance its biomass production. Therefore, the overall objective of this study was to clarify the structure and function of the *Wolffia* microbiome and to identify and characterize DABs acting as plant growth-promoting and -inhibiting bacteria (PGPB and PGIB, respectively) within *Wolffia* microbiome.

In Chapter 1, as the introduction of this study, *Wolffia* is highlighted as a next-generation bioresource with high potential for food and environmental applications. The chapter reviews current strategies to improve duckweed biomass production and summarizes existing knowledge on duckweed microbiomes and DABs, in order to clarify limitations and research gaps and to set the objectives of this study.

In Chapter 2, the structural and functional properties of the *Wolffia globosa* microbiome and its effects on host growth were investigated via co-cultivation with bacterial communities derived from various sources. The results suggested that *Wolffia* selectively assembles its microbiome members from surrounding environments. Although microbiome composition varied depending on microbial sources, a robust set of core taxa was conserved

across all sources. The microbiomes considerably influenced *Wolffia* growth, from strong promotion to clear inhibition. Interestingly, municipal wastewater–derived communities were the most effective in promoting *Wolffia* growth. Functional profiling of the microbiomes indicated that genes involved in chemotaxis, flagellar motility, biofilm formation, quorum sensing, secretion systems, and transporters facilitate bacterial colonization and adaptation to the rootless morphology of *Wolffia*. In addition, it was found that *Bdellovibrionaceae, Beijerinckiaceae*, and *Sphingomonadaceae* may act as "keystone taxa," shaping microbiome structure and directly or indirectly influencing *Wolffia* growth.

In Chapter 3, nearly 100 DABs were isolated from seven *Wolffia* microbiomes and individually co-cultivated with *W. globosa* for investigating their effect on the host growth. The results clarified that these DABs exert effects on *Wolffia* growth ranging from strong promotion to strong inhibition. Ten strains were identified as PGPB candidates and nine as PGIB candidates, of which seven PGPB and four PGIB corresponded to core ASVs, respectively. These results suggested that a small subset of core strains acts as positive or negative key players in the *Wolffia* holobiont. The balance of growth dynamics between beneficial and detrimental members is considered to determine the net community effects on host growth. Prominent promotive phenotypes of DABs occur across seven genera and are largely determined at the strain level, whereas inhibiting phenotypes were conserved at the genus level. The production of indole-related compounds emerges as a key physiological trait of DABs associated with growth outcomes.

In Chapter 4, the summary and conclusion of this study are given. Based on the insights gained from this study, perspectives and future challenges regarding practical approaches for microbiome engineering strategies to enhance the productivity of *Wolffia* biomass are proposed.

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Abbreviations

ANOVA: One-way analysis of variance

AS: Activated sludge

ASVs: Amplicon sequencing variants

CAS: Chrome azurol S

clr: Centered log ratio

DABs: Duckweed-associated bacteria

DF: Daughter frond

DO: Dissolved oxygen

DOC: Dissolved organic carbon

EC: Epidermal cells

EPG: Effect on plant growth

GF: Granddaughter frond

HCN: Hydrogen cyanide

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

IRCs: Indole-related compounds

KEGG: Kyoto Encyclopedia of Genes and Genomes

MA: Meristematic area

MF: Mother frond

MH: Modified Hoagland medium

PBS: Phosphate-buffered saline

PCoA: Principal-coordinate analysis

PCR: Polymerase chain reaction

PERMANOVA: Permutation multivariate analysis of variance

PGIB: Plant growth-inhibiting bacteria

PGPB: Plant growth-promoting bacteria

PW: Freshwater pond

RDA: Redundancy analysis

RGR: Relative growth rate

RP: Reproductive pocket

SE: Secondary effluent

TDN: Total dissolved nitrogen

WgAS: *Wolffia* co-cultivated with bacterial communities derived from activated sludge

WgPW: *Wolffia* co-cultivated with bacterial communities derived from freshwater pond

WgSE: *Wolffia* co-cultivated with bacterial communities derived from secondary effluent

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Chapter 1 Introduction

1.1 Duckweed

Duckweed is a common name of monocotyledonous floating aquatic plants belonging to the family Lemnaceae. Duckweeds are the smallest and fastest-growing flowering plants, and globally found in freshwater habitats, particularly slowly moving water or wetlands (Ali et al., 2016; Les et al., 2002; Ware et al., 2023). The family comprises five genera divided into two subfamilies, characterized by morphology and anatomy: Lemnoideae with roots (including *Landoltia, Lemna*, and *Spirodela*), and Wolffioideae without roots (including *Wolffia* and *Wolffiella*) (Fig. 1.1). As of the latest update, these five genera consist of a total of 36 species (Bog et al., 2020).

Morphologically, duckweeds have a highly simplified structure: a leaf-like structure, called a frond, ranges in size from less than 1 mm to 1.5 cm, and roots are present only in the subfamily Lemnoideae, with root number varying by genus. They reproduce both sexually and asexually, with vegetative propagation being the common reproductive mode (Jewell and Bell, 2023). Living duckweeds collected from all over the world are actively maintained at the Rutgers Duckweed Stock Cooperative (RDSC) at Rutgers University, New Brunswick, New Jersey, USA (http://www.ruduckweed.org/about.html).

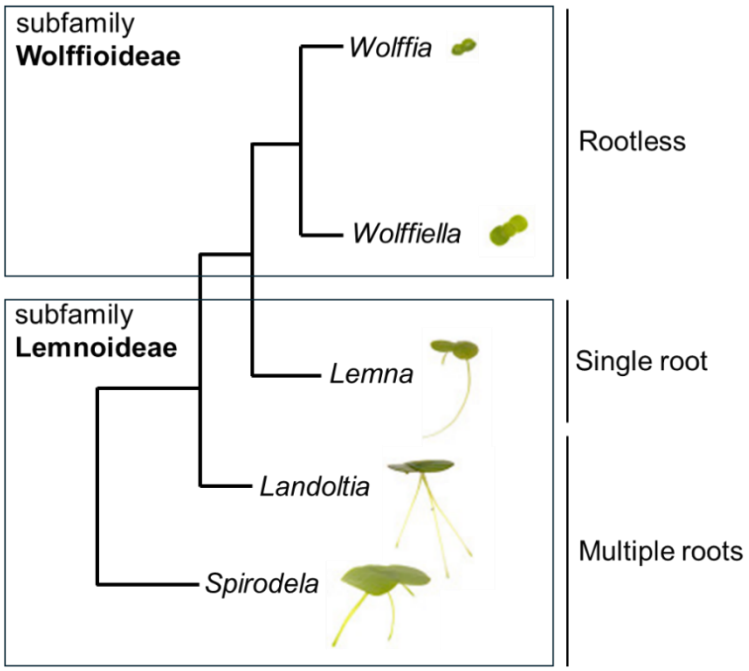


Fig. 1. 1 Structure of the family Lemnaceae (modified from Ware et al., 2023).

1.2 *Wolffia* as a specifically promising bioresource

Wolffia is a completely rootless genus within Lemnaceae and represents the smallest (0.5–1 mm) and fastest-growing plant among higher plants on earth (Bog et al., 2013; Lam and Michael, 2022). Figure 1.2 summarizes the biological features of *Wolffia*. The growth rate of *Wolffia* ranges from 0.155 to 0.559 d^{-1}, doubling in approximately 1–2 days (Sree et al., 2015; Ziegler et al., 2015). This genus includes 11 species and can be found globally across tropical to temperate regions, and thrives in various aquatic habitats, including freshwater and wastewater, under favorable conditions (Cheng and Stomp, 2009; Landolt, 1986; Sree and Appenroth, 2020). It is commonly known as watermeal and also locally in Thailand as *Khai-num* or *Phum* (Bhanthumnavin and McGarry, 1971; Bog et al., 2013).

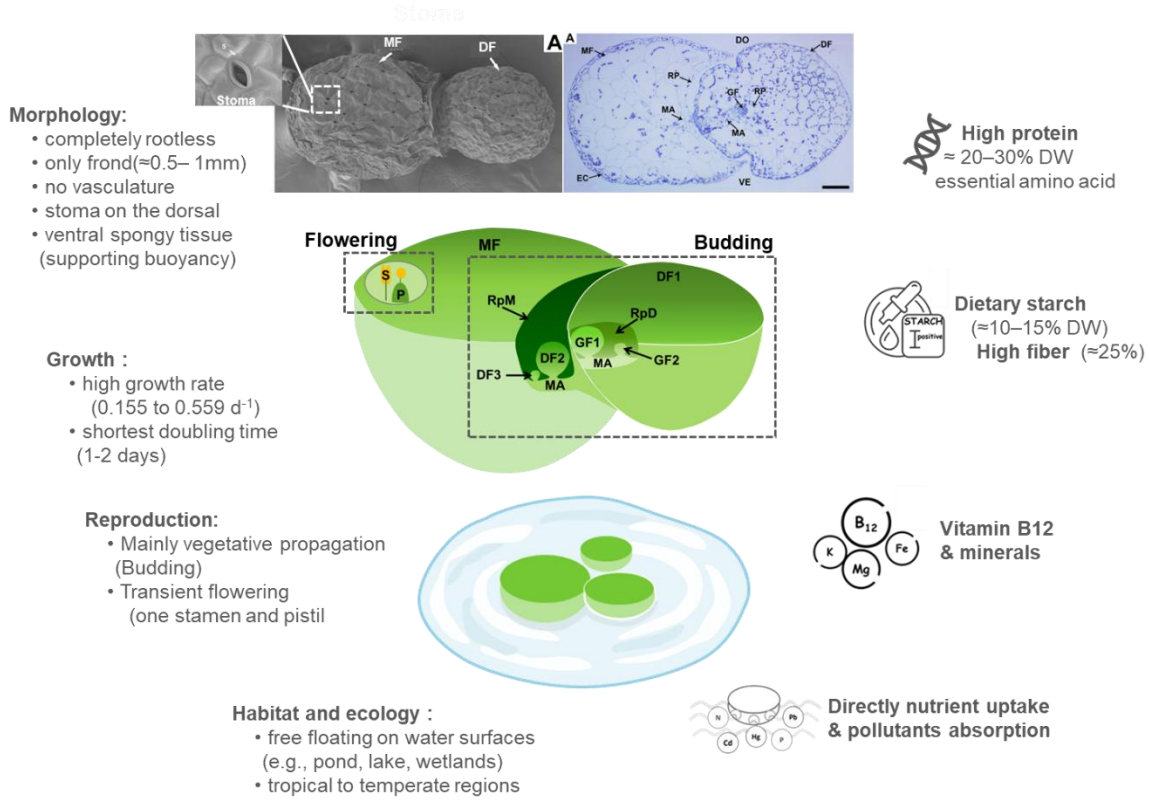


Fig. 1. 2 The important biological features of *Wolffia*. MF, mother frond; DF, daughter frond; GF, granddaughter frond; DO, dorsal part; MA, meristematic area; RpM, reproductive pocket of MF; RpD, reproductive pocket of DF; VE, ventral part; EC, epidermal cells. (modified from Yang et al. (2021)).

Unlike other members of the duckweed, *Wolffia* species have an extremely reduced and simplified morphology that eliminates complex structural investments (Fig. 1.2). The

most defining biological feature is its lack of roots. The plant body is reduced to a leaf-like structure called a frond that merges with a leaf and a stem, consisting of several thousand cells. Its unique and minimalist morphology is associated with a highly reduced set of genes in the auxin signaling and developmental pathways, particularly those involved in root development. These genetic reductions have driven the evolutionary degeneration of roots in *Wolffia* as a specific adaptation to its aquatic, free-floating lifestyle (Lam and Michael, 2022; Park et al., 2021). The resultant simple morphology confers an extremely high growth rate on *Wolffia* by channeling the majority of its metabolic energy directly into growth and reproduction.

In addition to its unique morphology and rapid proliferation, *Wolffia* biomass has an exceptionally nutritional value profile (Fig. 1.2). Its biomass is rich in protein (20 to 30% of its dehydrated mass) and contains all essential amino acids in adequate quantities for adults, which meet the standards set by the World Health Organization. The total starch content of biomass ranges from 10 to 15%, and the fiber content is around 25%, contributing to digestive health and supporting gut function through its dietary fiber. Fat (1–5%) is mainly polyunsaturated fatty acids, particularly α-linolenic acid (n-3 fatty acid), resulting in a very low omega-6 to omega-3 (n6/n3) ratio of less than 1. This favorable fatty acid profile is beneficial for health, particularly in vegetarians and vegans, as it may reduce the risk of various diseases and help address dietary imbalances (Appenroth et al., 2018; Duangjarus et al., 2022; Hu et al., 2022). Importantly, *Wolffia* contains plant-based bioavailable vitamin B12, which is rarely found in plants. In addition, it lacks calcium oxalate crystals, which are common in some other duckweed and terrestrial plants, which enhances the digestibility and safety of *Wolffia* as a food source (Acosta et al., 2024; Landolt and Kandeler, 1987; Mazen et al., 2004). Furthermore, *Wolffia* biomass contains various bioactive compounds, antioxidants (e.g., flavonoid, phenolic, anthocyanin, beta-carotene), and minerals (e.g., potassium, sodium, magnesium, calcium, iron, and zinc), and exhibits antimicrobial properties (Duangjarus et al., 2022; Kaplan et al., 2019; Monthakantirat et al., 2022; Yadav et al., 2024).

The multifaceted biological features of *Wolffia* underpin its diverse applications as an invaluable bioresource, including human food, animal feed, and feedstock for bioenergy and biomaterials, contributing to the bio-circular economy (Table 1.1, Fig. 1.3).

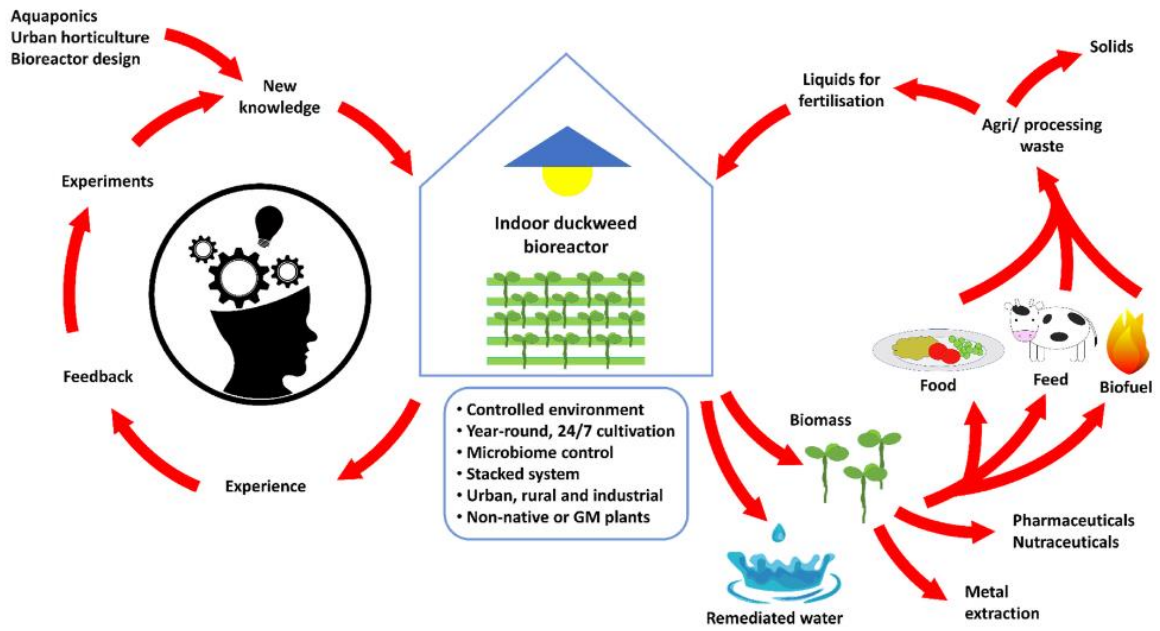


Fig. 1. 3 Infographic of the development and operation of duckweed cultivation systems, in line with the principles of the circular economy concept (Coughlan et al.,2022).

First, *Wolffia* has a longstanding history as an edible vegetable among local populations in Southeast Asian countries, including Thailand, Laos, and Myanmar (Bhanthumnavin and McGarry, 1971). Due to its relatively high tolerability to environmental stresses and pollutants, *Wolffia* can be cultivated in wastewater without supplementing chemical fertilizers. In addition, *Wolffia* has attracted attention as a "superfood" and novel functional food ingredient (Duangjarus et al., 2022; Kaplan et al., 2019; Monthakantirat et al., 2022; Yadav et al., 2024), and is now commercialized in various forms such as fresh vegetables, protein drinks, noodles, snacks, and dietary supplements, and is being positioned as an emerging food trend for health-conscious consumers worldwide (Saengthongpinit, 2017). Furthermore, *Wolffia* has been proposed as a promising crop species for protein production in space agriculture of bioregenerative life support systems for future space missions (Romano and Aronne, 2021; Romano et al., 2024).

Second, the use of *Wolffia* as a supplement in animal feed for chicken, swine, cattle, and fish not only enhances growth performance, egg quality, and nutritional intake (Anderson et al., 2011; Huque et al., 1996; Mwale et al., 2013), but also helps reduce reliance on land-

intensive feed crops. By integrating *Wolffia* into animal feed chains, it is therefore possible to promote more circular and resource-efficient production systems.

Table 1. 1 Potential applications of *Wolffia* biomass as a bioresource.

Application Field	Specific Uses & Products	Key Mechanisms & Benefits
Human Food & Nutrition	- Traditional consumption. - Fresh vegetables. - Commercial products: protein drinks, noodles, snacks, and supplements. - Functional food ingredients. - Protein production in space agriculture	- Nutritional Profile: High protein (20–30%), complete essential amino acids, dietary fiber, and rare plant-based Vitamin B12. - Health: Low n6/n3 fatty acid ratio, bioactive antioxidants, and absence of calcium oxalate. - Safety: High digestibility and lack of antinutrients.
Animal Feed	Dietary supplement for poultry (chicken), swine, and cattle Aquaculture feed (fish)	- Performance: Enhances animal growth performance, egg quality, and nutritional intake. - Sustainability: Reduces reliance on land-intensive feed crops (e.g., soybean).
Bioenergy & Bioproducts	- Feedstock for bioethanol and biogas production. - Feedstock for bioplastic production - Biofertilizer	- Substrate Quality: High carbohydrate/starch content allows for efficient fermentation. - Circular Economy: Valorizes biomass harvested from wastewater treatment processes.
Water Environmental Conservation & Management	Degradation of organic pollutants, including recalcitrant micropollutants Removal of nutrients and heavy metals	Degradation by plant and symbiotic microbes: Organic pollutants Adsorption and Uptake: Nitrogen, phosphorus, and heavy metals

Third, the *Wolffia* biomass rich in carbohydrate and other nutrients can be valorized as a feedstock for production of bioenergy (e.g., biogas and bioethanol), biofertilizer, and biomaterials (e.g., bioplastic) (Hubenova et al., 2023; Sowinski et al., 2019; Yoksan and Boontanimitr, 2023). Additionally, *Wolffia* functions as a biosorbent to remove organic pollutants, nutrients, and heavy metals in wastewater (Culley Jr et al., 1981; Farrell, 2012; Ritchie and Mekjinda, 2016). Therefore, *Wolffia* holds a potential contribution to a co-benefit system to combine wastewater purification and valuable bioresource production for future sustainable societies.

1.3 Strategies to enhance duckweed biomass production

The global duckweed market is valued at USD 89.4 million in 2025 and projected to reach USD 256.7 million by 2035, with an anticipated annual growth rate of 11.2% (https://www.futuremarketinsights.com/reports/duckweed-market). To meet the global demand increase and ensure product safety and quality, producers increasingly employ advanced cultivation and post-harvest techniques, such as precision farming, greenhouse and vertical production, and innovative sterilization methods, as well as approaches that harness plant–microbe interactions. Coughlan et al. (2022) have proposed a conceptual model for indoor duckweed bioreactor optimization, in which overall system performance is preceded by six key knowledge gaps that currently impede the development of large-scale indoor duckweed cultivation. These gaps concern (i) cultivation structures, (ii) cultivation conditions, (iii) media types, (iv) species and strains, (v) microbiome, and (vi) harvesting.

Among the proposed key knowledge gaps, recent research has increasingly focused on exploiting plant–microbe interactions to enhance duckweed biomass production, including the use of environmental microbial communities, selected beneficial microbes, and designed cultivation systems incorporating these microbes. For example, inoculation with environmental bacterial communities derived from freshwater ponds can enhance *Lemna minor* growth (up to around 14%) (Ishizawa et al., 2017). Another study comparing single strains with mixed communities has shown that both single isolates and bacterial communities significantly promote *L. minor* growth, with communities generally providing greater growth promotion (Laurich et al., 2024). Microbial communities originating from natural ponds increased *L. minor* growth by approximately 75% in pixel area and 30% in frond number, and improved plant fitness under zinc stress (O'Brien et al., 2020). Furthermore, a two-step cultivation process with *Acinetobacter calcoaceticus* P23 has been shown to enhance *L. minor* biomass production by approximately 1.9–2.3-fold (Ishizawa et al., 2020b). These examples demonstrate that harnessing plant–microbiome interactions through the use of selected beneficial strains or tailored microbial consortia is a promising strategy to enhance duckweed biomass production. Such microbiome-based approaches are cost-effective, eco-friendly, and highly reproducible, and therefore hold considerable promise for the sustainable intensification of duckweed cultivation systems, including *Wolffia*.

1.4 Duckweed microbiome

1.4.1 Plant microbiome and important constituents

Microbial communities associated with plants, collectively referred to as the "plant microbiome", colonize every accessible plant tissue, including both surface and internal tissues. The plant microbiomes strongly influence plant growth, health, and resilience by providing a range of vital functions (Compant et al., 2019; Trivedi et al., 2020). Plants and their microbiomes are therefore viewed as an integrated ecological unit, the "plant holobiont", within which continuous molecular communication and signal exchange shape both partners (Vandenkoornhuyse et al., 2015).

Microbial community composition differs among plant compartments (e.g. rhizosphere, endosphere, phyllosphere) and is structured in a non-random manner by host selection, environmental conditions, and microbe–microbe interactions (Coleman-Derr et al., 2016; Hamonts et al., 2018; Morella et al., 2020; Wang and Sugiyama, 2020). Within microbiomes, "core microbiome" members are the taxonomic groups that occur consistently across individuals, environments, or host genotypes and are thought to have key ecological functions and contribute to microbiome stability (Astudillo-García et al., 2017; Lemanceau et al., 2017; Yeoh et al., 2017; Zhou et al., 2024). Microbiomes also contain "hub" and "keystone" members, which play crucial roles in structuring and maintaining the whole microbial communities within the microbiomes. Hub taxa represent points of high interaction or correlation, acting as central mediators for the community organization, whereas keystone taxa are generally a small subset of the core microbiome and have disproportionately large influence on community structure, function, or host fitness relative to their abundance (Agler et al., 2016; Banerjee et al., 2018; Ishimoto et al., 2021; Li et al., 2024; Rawstern et al., 2023; Sun et al., 2024; Xie et al., 2025). The removal or loss of such keystone taxa can cause marked shifts in community composition and microbiome functions.

1.4.2 Current knowledge on duckweed microbiome

Similar to other plants, duckweeds strongly interact with microorganisms and selectively recruit bacterial populations from surrounding environments to shape their microbiomes. This relationship strongly influences duckweed growth, performance, and overall fitness (Fig. 1.4). Duckweeds release exudates and signal molecules and provide habitat that supports the activity of phylogenetically and functionally diverse duckweed-associated bacteria (DABs). In turn, DABs range from beneficial to detrimental partners: plant growth-promoting bacteria (PGPB) enhance growth, nutrient uptake, stress tolerance,

and pathogen suppression, whereas plant growth-inhibiting bacteria (PGIB) can lower duckweed growth and performance.

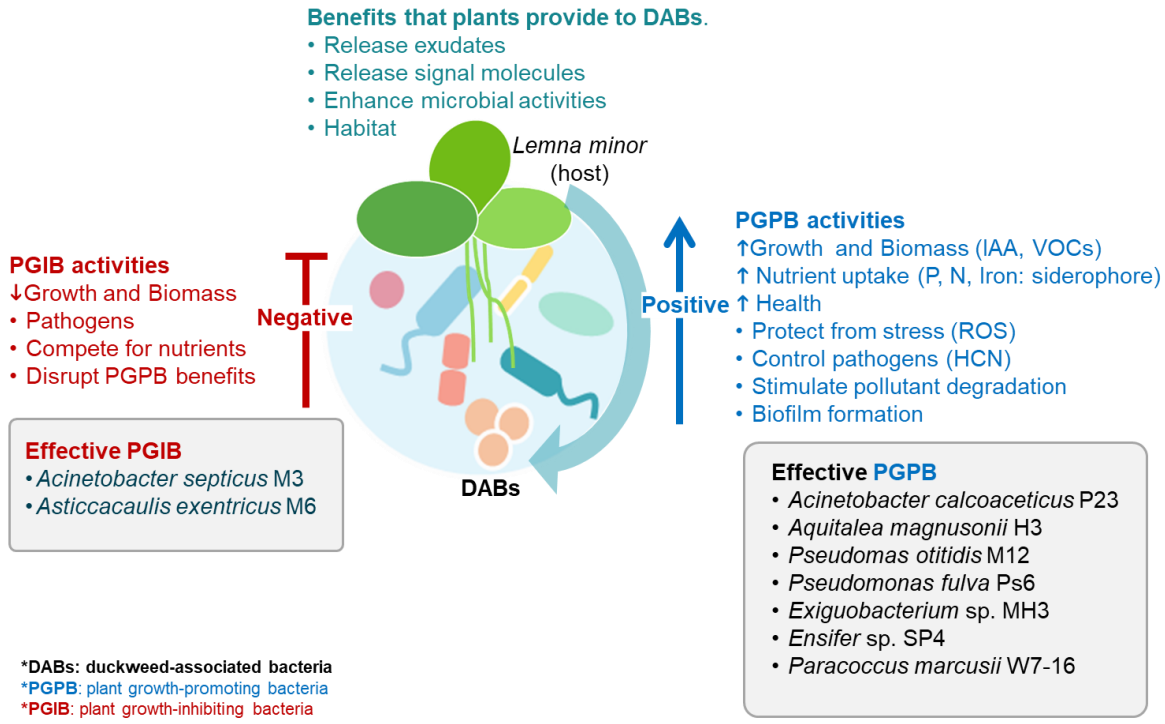


Fig. 1. 4 Overall duckweed microbiome.

Duckweed microbiome has been extensively investigated worldwide in both natural duckweed populations and inoculated duckweed, the latter established by co-cultivating duckweed with environmental microbial communities under laboratory conditions. Inocula commonly originate from freshwater ponds, activated sludge and secondary effluent from municipal wastewater treatment plants, and paddy soils. Across Asia, the USA, and Europe, researchers have employed culture-independent molecular methods such as libraries (Matsuzawa et al., 2010), denaturing gradient gel electrophoresis (Toyama et al., 2009), and 16S rRNA amplicon sequencing (Acosta et al., 2020; Bunyoo et al., 2022; Inoue et al., 2022a; Xie et al., 2015) to characterize the structure of the duckweed microbiome. More than 15 bioprojects based on 16S rRNA amplicon sequencing have been registered in the GenBank/DDBJ/EMBL databases, covering all five duckweed genera; however, most studies focus on rooted genera such as *Lemna* and *Spirodela* sp.

Previous studies, especially on rooted duckweed, have proven that the overall structure of the duckweed microbiome can vary between duckweed species (Bunyoo et al., 2022; Inoue et al., 2022a), between fronds and roots (Iwashita et al., 2020), and with the culture conditions

(Bunyoo et al., 2022). Environmental factors such as temperature, salinity, precipitation, pH, and water properties (concentrations of phosphorus and calcium) also influence bacterial richness and the biogeographic variation of duckweed-associated communities (Wei and Tan, 2023). In addition, the overall duckweed microbiome assembled depends on the microbial sources, while several core taxa were consistently present and conserved, including *Caulobacteraceae, Comamonadaceae, Methylophilaceae, Oxalobacteraceae, Pseudomonadaceae, Rhizobiaceae, Rhodospirillaceae*, and *Sphingomonadaceae* (Acosta et al., 2020; Bunyoo et al., 2022; Inoue et al., 2022a).

Meanwhile, a preliminary study by Inoue et al. (2022) and a study by Bunyoo et al. (2022) revealed some uniqueness in rootless duckweed microbiomes, such as small diversity compared with other duckweeds from the same location, and the enrichment of uncommon taxa (*Bdellovibrionaceae* and *Flavobacteriaceae*). Additionally, Xie et al. (2015) conducted the co-cultivation of *Wolffia australiana* with bacterial communities derived from paddy soils, demonstrating the assembly of unique bacterial groups on the plant surface, in contrast to those found on the leaves of terrestrial plants and submerged macrophytes.

Recent comparative studies revealed that, despite differences in habitat and life history between duckweeds and soil-based terrestrial plants, the phylogenetic structure of duckweed microbiomes closely resembles that of terrestrial plant microbiomes. Duckweeds preferentially and consistently host the members of Proteobacteria, similar to the phyllosphere and rhizosphere of terrestrial plants (Acosta et al., 2020; Ishizawa et al., 2020c). Comparisons of microbial communities between fronds and roots across three duckweed species further reveal organ-specific patterns (Iwashita et al., 2020).

1.4.3 Duckweed-associated bacteria influencing host plant growth

As the effects of microbiomes are ultimately caused by their constituents, studies have been made to isolate and characterize DABs from both natural and inoculated duckweeds, especially focusing on rooted duckweeds such as *Lemna* and *Spirodela*. To date, approximately 20 strains of DABs have been proven to function as PGPB, as summarized in Table 1.2. *A. calcoaceticus* P23 is a well-studied example and was described as the first PGPB isolated from *Lemna aequinoctialis* (formerly *Lemna aoukikusa*) (Yamaga et al., 2010). This strain significantly promotes the growth of *Lemna* spp. (Ishizawa et al., 2020b; Khairina et al., 2021; Yamakawa et al., 2018). Of the previously isolated PGPB, only two strains isolated from *S. polyrhiza* demonstrated the growth promotion of *Wolffia* (Yoneda et al., 2021).

Meanwhile, four strains of DABs were identified to function as PGIB, including *Acinetobacter ursingii* M3, *Asticcacaulis excentricus* M6, *Pannonibacter phragmitetus* W9-8, *and Stenotrophomonas maltophilia* W9-6. Notably, the apparent PGPB effect of certain strains, such as *A. calcoaceticus* P23, can shift to growth inhibition under low-nitrogen conditions, likely due to competition for minerals between microbes and host plants (Khairina et al., 2021). These observations underscore that PGPB performance depends on host species, environmental context, and the indigenous microbiome.

Functionally, DABs isolated to date have proven to possess multiple traits affecting duckweed growth (Fig. 1.4). However, the roles of those traits in duckweed growth remain partly controversial. For example, several PGPB can produce indole-3-acetic acid (IAA), whereas exogenous application of IAA did not promote *L. minor* growth (Utami et al., 2018). By contrast, Ishizawa et al. (2017) demonstrated that phosphate-solubilizing activity was positively correlated with duckweed growth promotion, even though sufficient phosphorus in the soluble form was supplied in the cultivation systems. A possible explanation is that DABs supply phosphate to the host through the breakdown of dead bacterial cells, plant exudates, and phosphate salts in the medium. Overall, potential growth-promoting mechanisms exerted by PGPB include phytohormone modulation (e.g. IAA), biological nitrogen fixation (Makino et al., 2022), phosphate solubilization, potassium solubilization (Boonmak et al., 2023), and siderophore-mediated iron supply. In addition to these common mechanisms, some PGPB can promote the growth of duckweed through enhancing its tolerance to salinity and heavy-metal toxicity (Tang et al., 2015). Also, *A. calcoaceticus* P23 can promote duckweed growth in the presence of phenol, a common aromatic pollutant, by efficient degradation ability of phenol to lower its toxic effect (Khairina et al., 2021; Yamaga et al., 2010). PGPB can further protect duckweeds from pathogens via nutrient competition, antibiosis, and enzymatic degradation of cell walls. By contrast, PGIB suppresses duckweed growth without causing visible pathogenic symptoms; their mechanisms remain unclear, but nutrient competition and disruption of PGPB benefits are likely contributors (Ishizawa et al., 2019; Ishizawa et al., 2017).

Table 1.2 List of DABs isolated from duckweed.

Strain	Isolation sources	Functional roles in duckweed growth	Plant growth-promoting properties	Reference
PGPB				
Acinetobacter calcoaceticus P23	*L. aequinoctialis* enriched in phenol	Promotes the growth of *L. aequinoctialis* with or without phenol, increases host chlorophyll content, and degrades phenol with the host.	Phosphate solubilization	Yamaga et al. (2010); Suzuki et al. (2014); Toyama et al. (2017)
Exiguobacterium sp. MH3	Rhizosphere of *L. minor*	Promotes *L. minor* growth and enhances Cr(VI) removal under stress.	Siderophore production; IAA production	Tang et al. (2015)
Aquitalea magnusonii H3	*L. minor* co-cultured with freshwater pond water	Promotes *L. minor* growth and overcomes growth inhibition by PGIB through competitive colonization.	IAA production; phosphate solubilization; siderophore production; hydrogen cyanide production	Ishizawa et al. (2017, 2019)
Pseudomonas otidis M12	*L. minor* co-cultured with freshwater pond water	Accelerates *L. minor* growth.	IAA production; phosphate solubilization; siderophore production; hydrogen cyanide production	Ishizawa et al. (2017a, b)
Pseudomonas fulva Ps6	*L. minor* co-cultured with freshwater pond water	Promotes L. minor growth by increasing frond number and competes with P23 at later stages.	IAA production; phosphate solubilization	Yamakawa et al. (2018)
Chryseobacterium sp. 27AL	*L. gibba* cultivated in non-sterile wastewater from a food factory	Promotes *L. gibba* growth under both rich- and low-nitrogen conditions (e.g. food factory wastewater) without competing with the host for nitrogen.	IAA production; siderophore production	Khairina et al. (2021)
Luteitalea sp. TBR-22	Fronds of wild duckweed (*L. minor*)	Broadly promotes the growth of six duckweed species (*L. aequinoctialis*, *L. minor*, *S. polyrhiza*, *Landoltia punctata*, *Wolffia arrhiza*, and *Wolffia globosa*).	–	Yoneda et al. (2021)

Strain	Isolation sources	Functional roles in duckweed growth	Plant growth-promoting properties	Reference
Paludibaculum sp. F-183	Fronds of wild duckweed (*S. polyrhiza*)	Broadly promotes the growth of six duckweed species (*L. aequinoctialis*, *L. minor*, *S. polyrhiza*, *Landoltia punctata*, *W. arrhiza*, and *W. globosa*).	IAA production	Yoneda et al. (2021)
Aquidulcibacter sp. SP-2-C10	Frond of *S. polyrhiza*	Promotes *L. minor* growth in sterile medium and secondary sewage effluent.	IAA production; siderophore production	Iwashita et al. (2021)
Ensifer sp. SP4	*S. polyrhiza* co-cultured with municipal wastewater effluent	Enhances *S. polyrhiza* biomass via increased nitrogen metabolism and photosynthesis.	Siderophore production	Toyama et al. (2022)
Pelomonas sp. MRB1	*Lythrum anceps*	Promotes *L. minor* growth by increasing frond number, dry weight, and chlorophyll content.	IAA production; nitrogen fixation	Makino et al. (2022)
Rhodanobacteraceae sp. MRB2	*Lythrum anceps*	Promotes *L. minor* growth by increasing frond number, dry weight, and chlorophyll content.	IAA production; siderophore production	Makino et al. (2022)
Pelomonas sp. MRB3	*Lythrum anceps*	Promotes *L. minor* growth by increasing frond number, dry weight, and chlorophyll content.	IAA production; siderophore production; nitrogen fixation	Makino et al. (2022)
Bradyrhizobium sp. MRB4	*Lythrum anceps*	Promotes *L. minor* growth by increasing frond number, dry weight, and chlorophyll content.	IAA production; nitrogen fixation	Makino et al. (2022)
Pelomonas saccharophila MRB3	Rhizosphere of *Lythrum anceps*	Releases NH_4^+ and PO_4^{3-} through spontaneous lysis, supplying nutrients for duckweed.	–	Ishizawa et al. (2023)
Shinella curvata W12-8	*L. aequinoctialis* after enrichment in wastewater	Promotes *S. polyrhiza* growth by increasing frond number and dry weight.	IAA production; phosphate solubilization; siderophore production; potassium solubilization	Boonmak et al. (2024)
Paracoccus marcusii W7-16	*S. polyrhiza* after enrichment in wastewater	Promotes *S. polyrhiza* growth by increasing frond number and dry weight.	IAA production; phosphate solubilization; siderophore production	Boonmak et al. (2024)

Strain	Isolation sources	Functional roles in duckweed growth	Plant growth-promoting properties	Reference
Novosphingobium subterraneum W5-13	*S. polyrhiza* after enrichment in wastewater	Promotes *S. polyrhiza* growth by increasing frond number and dry weight.	IAA production; phosphate solubilization; siderophore production	Boonmak et al. (2024)
Acidovorax kalamii W7-18	*S. polyrhiza* after enrichment in wastewater	Promotes *S. polyrhiza* growth by increasing frond number and dry weight.	IAA production; phosphate solubilization; siderophore production	Boonmak et al. (2024)
Kinneretia sp. KLaR20	*L. aequinoctialis* grown naturally in food-factory treated wastewater	Promotes *L. aequinoctialis* growth by increasing frond number and dry weight and inhibits the growth of harmful microalgae.	IAA production; siderophore production	Thanh Phạm et al. (2025)
PGIB				
Acinetobacter ursingii M3	*L. minor* co-cultured with freshwater pond water	Inhibits the growth of *L. minor*.	Phosphate solubilization; siderophore production	Ishizawa et al. (2017)
Asticcacaulis excentricus M6	*L. minor* co-cultured with freshwater pond water	Inhibits the growth of *L. minor*.	IAA production; siderophore production; hydrogen cyanide production	Ishizawa et al. (2017)
Pannonibacter phragmitetus W9-8	*S. polyrhiza* after enrichment in wastewater	Inhibits the growth of *S. polyrhiza*.	nd	Boonmak et al. (2024)
Stenotrophomonas maltophilia W9-6	*S. polyrhiza* after enrichment in wastewater	Inhibits the growth of *S. polyrhiza*.	nd	Boonmak et al. (2024)

nd, no data available.

1.5 Objectives of this study

Amid a rapidly growing global population, challenges related to food security and environmental sustainability are intensifying. *Wolffia* emerged as a valuable bioresource for a potential solution in this context. Previous studies have demonstrated that duckweed microbiomes and DABs can enhance biomass production, improve nutritional profiles, and support phytoremediation performance of duckweeds. However, most of these studies have focused on rooted genera, and comparative surveys have largely excluded the rootless genera *Wolffia* and *Wolffiella*, with only a few reports providing preliminary observations. Consequently, the microbiomes of rootless duckweed remain poorly understood and have not yet been systematically investigated.

Given their extreme morphological reduction and lack of roots, it is still unclear whether rootless duckweeds share similar phylogenetic microbiome structures with rooted duckweeds and terrestrial plants. Moreover, no DABs have yet been isolated from *Wolffia*. Although one study reported growth promotion of *Wolffia* by two DAB strains isolated from *S. polyrhiza*, several studies have suggested that PGPB performance depends strongly on host species, environmental context, and the indigenous microbiome. These observations highlight the need to isolate and evaluate well-matched strains specifically for rootless duckweeds, particularly *Wolffia*. Overall, these knowledge gaps limit efforts to harness plant–microbiome interactions and beneficial bacteria to improve *Wolffia* productivity. Therefore, this study aims to 1) characterize the structural and functional microbiome of rootless duckweed, 2) isolate DABs specific to *Wolffia* and evaluate their effects on *Wolffia* growth individually, and 3) characterize the physiological traits of DABs that may be associated with *Wolffia* growth.

Chapter 1 provides a general introduction of duckweed, the biological features of *Wolffia* as a bioresource, strategies to enhance duckweed production, and the duckweed microbiome. Based on the review of previous studies, the objective of this study was proposed as mentioned above.

In Chapter 2, the phylogenetic structure and functions of the microbiome of *Wolffia* and its effects on *Wolffia* growth are examined through *W. globosa* co-cultivated with bacterial communities derived from freshwater ponds, secondary effluent, and activated sludge. Based on the results obtained, the factors shaping microbiome assembly are characterized. This chapter aims (i) to assess how different bacterial communities affect the growth of *Wolffia*;

(ii) to clarify the structure and functions of the bacteria associated with *Wolffia* and its core taxa; and (iii) to determine what factors cause variations in the *Wolffia* microbiome.

In chapter 3, nearly 100 DAB strains are isolated from seven *Wolffia* microbiomes, and their individual effects on host growth, taxonomic identification, and physiological traits associated with host growth were evaluated. This chapter aims to assess the effects of DABs isolated from *Wolffia* microbiome on host plant growth and characterize the physiological traits that may be associated with this growth.

In Chapter 4, the findings of this study were summarized. The chapter also provided conclusions, future research directions, and perspectives on the application of *Wolffia*–microbiome interactions.

Chapter 2 Structural and functional properties of bacterial communities associated with rootless duckweed (*Wolffia globosa*) and their effect on the *Wolffia* growth

2.1 Introduction

As mentioned in Chapter 1, extensive studies on rooted duckweeds have proven that duckweed microbiomes significantly affect duckweed growth, performance, and overall fitness. In addition, a few preliminary reports previously revealed that rootless duckweeds form unique microbiomes that differ from those of rooted duckweeds. Nevertheless, the microbiomes on rootless duckweeds have not been systematically characterized. Moreover, the effects of entire microbial communities on rootless duckweeds have not been rigorously evaluated. Given that rootless duckweeds, particularly *Wolffia,* are a promising next-generation bioresource, it is important to understand the characteristics and functions of the *Wolffia* microbiome that can affect their growth. Therefore, this chapter aims (i) to assess how different bacterial communities affect the growth of *Wolffia*; (ii) to clarify the structure and functions of the bacteria associated with *Wolffia* and its core taxa; and (iii) to determine which factors cause changes in the *Wolffia* microbiome. To these ends, this chapter conducted co-cultivation of axenic *W. globosa* with 18 different bacterial communities derived from various sources, including ten freshwater ponds, four municipal secondary effluents and four activated sludges from municipal wastewater treatment plants, and systematically characterized the structure and functions of the *Wolffia* microbiomes using a 16S rRNA amplicon sequencing and diverse analysis methods.

2.2 Materials and methods

2.2.1 Recovery of bacterial communities from various sources

As the microbial source, surface water samples were collected from 10 freshwater ponds in September 2023, along with secondary effluent and activated sludge samples from 4 municipal wastewater treatment plants in Osaka, Japan, in December 2023. The locations of sampling points are shown in Table 2.1. The activated sludge samples were pretreated to disperse the flocs as follows: the samples were left at room temperature to allow the sludge flocs to settle. After settling, 2.5 g-wet of the settled sludge was added to 250 mL of phosphate-buffered saline (PBS), shaken at 180 rpm and 28 °C overnight. To recover the

bacterial communities from pond water, secondary effluent, and pretreated activated sludge, all samples were passed through a 3.0 μm membrane filter (MF-Millipore^{TM}, Merk Millipore, Darmstadt, Germany) to remove coarse particles, including fungi, microalgae, and larger activated sludge flocs. Sixty milliliters of the filtrate were then centrifuged at 10,000×*g* for 10 min at 4 °C to recover the bacterial communities. The bacterial communities were washed twice with a sterile modified Hoagland (MH) medium (Toyama et al., 2006) and then re-suspended in the original volume of sterile MH medium for the co-cultivation experiment. The bacterial communities recovered from pond water, secondary effluent, and activated sludge were hereinafter referred to as categories PW, SE, and AS, respectively.

Table 2. 1 Locations of sampling points and water properties.

Sample ID	Sample name	Temperature (°C)	pH	Water properties (mg/L)						
				Original sources				Medium after 10 days of cultivation		
				DO	DOC	TDN	PO_4-P	DOC	TDN	PO_4-P
PW_A	Kokoroji pond	29.2	7.52	7	4.75	0.28	<0.05	27.83	0.17	0.05
PW_B	Komoga pond	28.7	6.83	7.7	7.72	0.43	<0.06	32.80	0.17	0.07
PW_C	Library pond	28.7	6.65	7.8	7.22	0.39	<0.07	52.00	0.20	0.05
PW_D	Uzuwa pond	31.0	7.54	6.8	13.2	0.23	<0.08	40.75	0.18	0.08
PW_E	Inukai pond	32.0	8.54	10.1	6.01	0.25	<0.09	27.37	0.17	0.07
PW_F	Kasugao pond	30.2	6.89	7.7	3.50	0.77	<0.10	19.15	0.19	0.06
PW_G	Ushikgakubi pond	28.7	6.83	3.8	5.49	0.47	<0.11	31.07	0.20	0.06
PW_H	Ouji pond	30.7	9.13	12.1	5.24	0.43	<0.12	34.43	0.26	0.07
PW_I	Hasumaga pond	30.3	7.66	9.2	7.27	1.42	<0.13	23.21	0.19	0.06
PW_J	Zuion pond	31.4	7.21	4.4	5.26	0.60	<0.14	21.95	0.16	0.06
SE_1	Minami-Suita city	NT	NT	NT	6.01	2.06	1.27	12.99	1.07	<0.05
SE_2	Ebie city	NT	NT	NT	7.38	6.12	0.82	9.43	1.00	<0.05
SE_3	Ikeda city	NT	NT	NT	4.81	2.31	0.81	6.01	0.78	<0.05
SE_4	Jahachijo city	NT	NT	NT	5.85	4.13	0.25	5.63	1.00	<0.05
AS_1	Minami-Suita city	NT	NT	NT	6.70	1.67	2.90	0.10	0.00	<0.05
AS_2	Ebie city	NT	NT	NT	8.44	2.37	2.20	7.72	0.39	<0.05
AS_3	Ikeda city	NT	NT	NT	9.07	2.99	2.25	12.89	1.08	<0.05
AS_4	Jahachijo city	NT	NT	NT	9.75	2.77	2.32	13.42	0.64	<0.05

*NT=Not determine

2.2.2 Co-cultivation of Wolffia with bacterial communities

Axenic *W. globosa* (clone no. RDSC8152) was used as the test plant. The plant was sterilized with sodium hypochlorite, and the sterilized plant was maintained as previously described (Ishizawa et al., 2021). We have verified the absence of microorganisms routinely and prior to use in the co-cultivation experiments by confirming no microbial growth occurred

on R2A agar “DAIGO” (Shiotani M.S. Co., Ltd., Hyogo, Japan) after applying sterile *W. globosa* with and without homogenization to the plates. The plants were cultured in MH medium for 5 days prior to the experiment. Twenty fronds of the precultivated *Wolffia*, which included 10 fronds from attached mother-daughter pairs and 10 single fronds, were transferred into a flat-bottomed 6-well plate containing 10 mL of bacterial community suspensions. The inoculated fronds occupied an area of approximately 0.29–0.30% of the well surface. The plates were then cultured for 10 days in a growth chamber set to 28 °C, with a light intensity of 80 μmol/m^2/s, and a photoperiod of 16 h light/8 h dark. The cultivation period was divided into two stages: i) the colonization stage and ii) the growing stage. The colonization stage lasted for the first 5 days to allow the bacterial community to attach to and colonize the *Wolffia*. Evidence suggests that 5 days is sufficient for the duckweed microbiome to stabilize (Acosta et al., 2020; Inoue et al., 2022a; Ishizawa et al., 2020c). Subsequently, all *Wolffia* were transferred to 10 mL of fresh bacteria-free MH medium to continue the growing stage. During this stage, the MH medium was renewed on day 8 to address nutrient deficiency. After 10 days of cultivation, the *Wolffia* were collected, excess liquid on the fronds was blotted with sterile tissue paper, and the fronds were placed in sterile microtubes for further experiments. The medium was also collected for water quality measurements. Axenic *W. globosa* cultivated in MH medium without any bacterial communities served as experimental control. We performed five repetitions for each sample and the control.

2.2.3 Evaluation of Wolffia growth

To measure the growth of *Wolffia*, the frond area was periodically recorded at 0, 3, 5, 7, and 10 days. We ensured consistency by photographing using the same equipment and maintaining the same distance, lighting, and time. The photos were analyzed using Collaboratory Software (https://colab.research.google.com; accessed in September 2023) to detect the green parts of the plant and calculate the relative growth rate (RGR). In brief, the analysis process involved converting the images from BGR to HSV color space. A mask was applied to isolate the green areas of the plant, and we counted the number of white pixels in the masked image that represented these green areas. This count was then used to determine the percentage of green pixels compared to the entire surface of the image, which reflects the frond area of *Wolffia*. The percentage of the green area was subsequently used to calculate *Wolffia* RGR. The increase or decrease in plant growth was assessed using the effect on plant growth (EPG) value, which is derived from the RGR compared to the control, and the EPG values greater than +5%, between –5% and +5%, and less than –5% were judged as promoting,

neutral, and inhibitory effects, respectively, as previously described (Ishizawa et al., 2017). The EPG was calculated as follows:

$$EPG\ (\%) = \frac{G(T) - G(C)}{G(C)} \times 100$$

where *G(T)* is the mean of RGR, calculated from the *Wolffia* area in the co-cultivation with bacterial community after 10 days of cultivation, and *G(C)* is that of the axenic *Wolffia* served as the control. Here, standard deviation (SD) for EPG was calculated as:

$$SD(EPG) = \frac{\sqrt{\left(SD\left(G(T)\right)^2 + SD\left(G(C)\right)^2\right)}}{G(C)} \times 100$$

where *SD(G(T))* and *SD(G(C))* are the standard deviation of *G(T)* and *G(C)*, respectively.

2.2.4 Determination of physicochemical water quality parameters

Water temperature and pH were measured for the collected water at the sampling points using a portable pH meter (TPX–999i; Toko Kagaku, Tokyo, Japan). Dissolved oxygen (DO) was measured with a portable galvanic DO sensor (DO–30; Kasahara Chemical Instruments, Saitama, Japan). Additionally, dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and phosphorus as phosphate (PO_4–P) in the collected water and the medium after co-cultivation experiments were analyzed as environmental parameters. Samples were filtered through a 0.45 μm cellulose acetate filter (Advantec, Tokyo, Japan) before analysis. DOC and TDN were measured using a total organic carbon analyzer (TOC–L, Shimadzu, Kyoto, Japan) equipped with a total nitrogen measuring unit (TMN–L, Shimadzu). PO_4–P was analyzed using ion chromatography (IC) with a Shimadzu SLC-10Avp system (Shimadzu), which included a CDD-10Asp detector (Shimadzu) and an IonPac AS4A-SC column (4×250 mm, Thermo Scientific, Massachusetts, USA). The eluent was composed of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$, and delivered at a flow rate of 1.0 mL/min. The water properties of the original microbial sources and MH medium after 10 d of cultivation are included in Table 2.1.

2.2.5 DNA extraction and 16S rRNA amplicon sequencing

Indigenous bacterial communities from the original microbial sources were filtered through a 0.2 μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan). The filtrates were subjected to DNA extraction. After 10 days of co-cultivation, *Wolffia* samples were homogenized using a BioMasher II (Nippi, Tokyo, Japan) for DAN extraction. Microbial DNA from all samples was extracted using the Cica Geneus DNA Extraction ST kit (Kanto Chemical,

Tokyo, Japan). The extracted DNA was subjected to 16S rRNA amplicon sequencings targeting the V5-V6 region of the bacterial 16S rRNA gene with primers 799F (Chelius and Triplett, 2001) and 1185mR (Hodkinson and Lutzoni, 2009). Sequencing was performed on an Illumina MiSeq sequencer (Illumina, CA, USA) at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan).

2.2.6 Data processing and analyses

The raw reads from 36 samples were processed using the QIIME2 version 2023.7 pipeline, which included 18 original microbial source samples designated S_PW_ (with letters A-J for category PW) and S_SE_ and S_AS_ (with numbers 1-4 for categories SE and AS). Eighteen *Wolffia* microbiome samples were designated as WgPW_, WgSE_, and WgAS_, using the same letter and number designations as their corresponding original microbial source samples. The obtained sequences were processed using QIIME 2.0; the raw reads were demultiplexed and filtered, high-quality reads were merged, and chimeric sequences were removed. The complied high-quality sequences were clustered into amplicon sequencing variants (ASVs) using the DADA2 plugin (Callahan et al., 2016). Then, ASVs were assigned to a taxonomy by the QIIME2 feature-classifier plugin based on the SILVA SSU Ref NR database v138. ASVs whose sequences were not present in the database and cyanobacterial ASVs were excluded. Finally, an abundance table combined with taxonomy information was generated. Rarefaction analysis was performed based on the feature table, with random sampling to the minimum sequencing depth (19,180) of all samples using the q2-diversity plugin. The Shannon and Chao1 indices were used as an index of alpha diversity, and one-way analysis of variance (ANOVA) with Honestly Significant Difference (Tukey HSD) test was conducted for assessing statistical differences. Beta diversity of bacterial communities was analyzed using principal-coordinate analysis (PCoA) based on the Bray-Curtis matrix in R version 4.3.2 with the "vegan" package to visualize the compositional dissimilarity in community structure between groups of samples. Permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2017) was conducted to assess the statistical significance of the observed differences in microbiome structure between groups. Data were visualized using the "ggplot2," "VennDiagram," and "ComplexHeatmap" packages in R.

To explore the environmental factors or bacterial families that drive the structural variation in *Wolffia* microbiomes and the original microbial sources, redundancy analysis (RDA) and partial RDA were conducted using the "vegan" package in R (Legendre and Legendre, 2012). Forward selection was performed with the "forward.sel" function from the "packfor" package to identify the most significant explanatory variables in relation to the

microbiome structure (Dray et al., 2013). Parameters included 32 explanatory variables, comprising 3 environmental factors (DOC, TDN, and PO_4–P), and 29 bacterial families with a relative abundance greater than 5%. The feature table was transformed using the Hellinger transformation, and the explanatory variables were standardized prior to the analysis. Variance inflation factors (Legendre and Legendre, 2012) were calculated for the variables from the RDA using the "vif.cca" function in the vegan package to detect redundant or multicollinear variables with a recommended threshold of below 10. Permutation testing of the model was performed with 999 permutations. The significance of the model and explanatory factors was determined based on the *p* values adjusted through the permutation-based approach inherent in the "anova.cca" function, with adjusting the p values < 0.05 considered statistically significant.

Functional profiling of the original microbial sources and *Wolffia* microbiomes was predicted based on ASVs using the PICRUSt2 algorithm (Douglas et al., 2020) through QIIME2. The predicted functional genes were categorized into pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (Kanehisa and Goto, 2000). For gene categories, functions with a mean relative abundance >0.4% were categorized into tier 3, normalized among the 36 communities, and the z-scores were plotted using the "ComplexHeatmap" package in R.

To identify the core microbiome of *Wolffia*, the ASVs with an occurrence frequency greater than 0.7 (Wang et al., 2023) in each *Wolffia* category (i.e., WgPW, WgSE, and WgAS), regardless of their relative abundance, were defined as core *Wolffia* taxa. Data were transformed using centered log ratio (clr) transformation, where the abundance counts were compared to their geometric mean. Taxa with clr values close to 0 indicated their average abundance. The transformed data were displayed using the "ComplexHeatmap" package in R.

The potential key players influencing *Wolffia* growth were identified based on its relationship with the relative abundance of bacterial families. Pearson correlation was constructed using the "metan" package in R and visualized using the "corrplot" package based on hierarchical clustering. Positive and negative correlations with *Wolffia* growth were then visualized as scatter plots.

2.3 Results

2.3.1 Effect of bacterial communities on Wolffia growth

The RGR of axenic *Wolffia* without any bacterial communities (the experimental control) was 0.328±0.015 day^{-1} under the experimental settings of this study. Bacterial communities recovered from three categories of microbial sources (PW, SE, and AS) had different effects on *Wolffia* growth, namely inhibitory, neutral, or promoting effects (Fig. 2.1). Six of ten communities in the category PW had neutral effects on *Wolffia* growth, while three (PW_E, PW_F, and PW_H) reduced growth by up to –12.2% without visible disease symptoms or chlorosis (Fig. 2.2). Only community PW_A in this category showed a slight promoting effect (+5.3%), marginally exceeding the threshold for promotion (+5%). In contrast, communities from AS and SE categories significantly promoted growth, with the EPG ranging from +21% to +55.6%, resulting in *Wolffia* growth of up to 1.6-fold than those from PW.

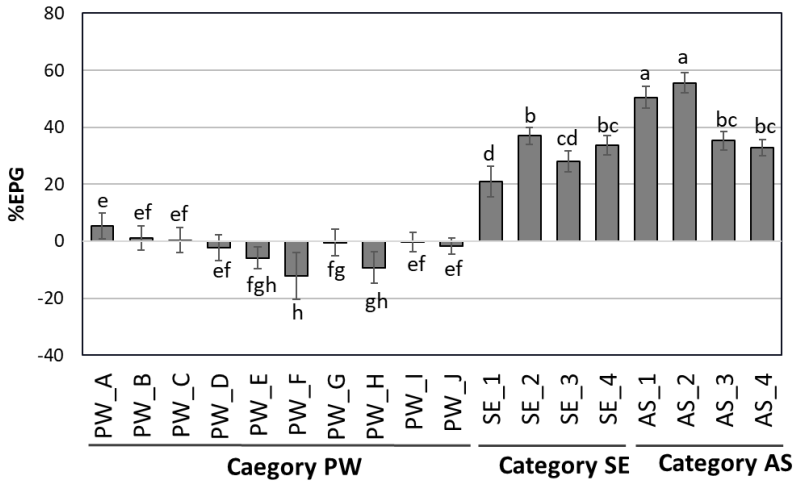


Fig. 2. 1 Effects on plant growth (EPG) of bacterial communities from categories PW, SE, and AS for 10-day cultivation term. Error bars represent standard deviations (n = 5). Bars marked with different letters indicate significant differences ($p < 0.05$, Tukey's HSD test). Letters a–h denote EPG ranks from highest to lowest.

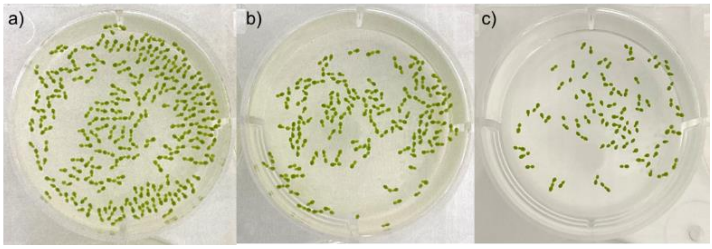


Fig. 2. 2 Representative appearance of *W. globosa* affected by bacterial communities after 10 days of cultivation: a) Promoting effects, b) Control, and c) Inhibitory effect.

This study used a two-stage cultivation system, allowing bacterial colonization before transferring *Wolffia* to a fresh axenic medium, with medium renewal on day 8 to prevent nutrient depletion that could lead to turion production. Monitoring *Wolffia* growth from day 5 to day 10 revealed a shift in SE communities that transitioned from inhibitory to promoting effects, while AS communities consistently maintained the growth-promoting effects (Fig. 2.3)

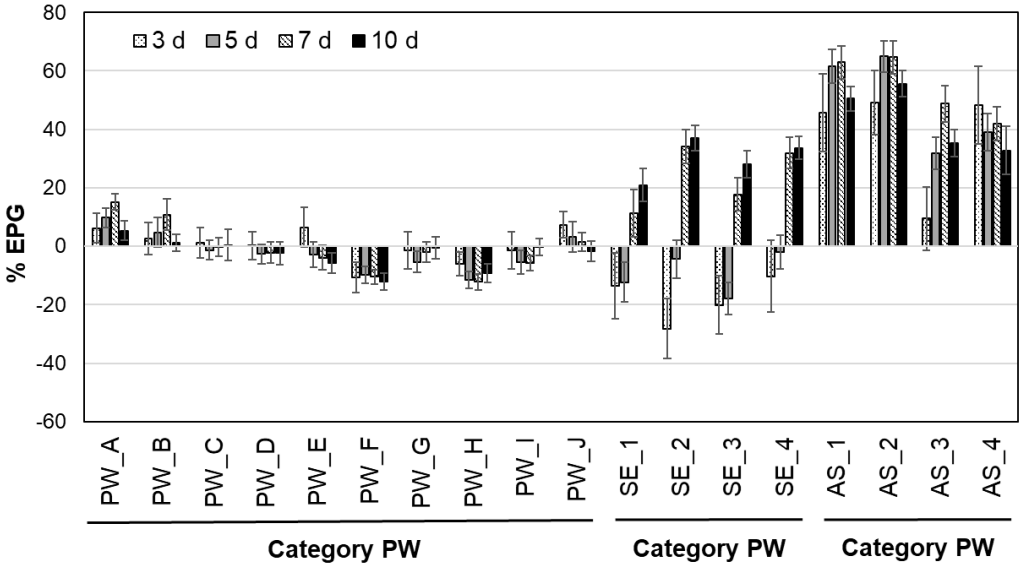


Fig. 2. 3 Effects on plant growth (EPG) of bacterial communities from categories PW, SE and AS on *W. globosa* growth at 0, 3, 5, 7 and 10 days of cultivation relative to the control.

2.3.2 Diversity and taxonomic composition of Wolffia microbiome in comparison with bacterial communities in microbial sources

Through 16S rRNA amplicon sequencing of 18 microbial sources and 18 *Wolffia* microbiomes, 1,071,728 reads were obtained, identifying 5,699 ASVs (Table S2, S3). Among the microbial sources, SE had the highest number of ASVs (2,847), followed by PW (1,504) and AS (1,102). The *Wolffia* microbiomes contained 1,077 ASVs, with only 1.7% to 5.5% overlapping with those from their microbial sources (Fig. 2.4).

a)

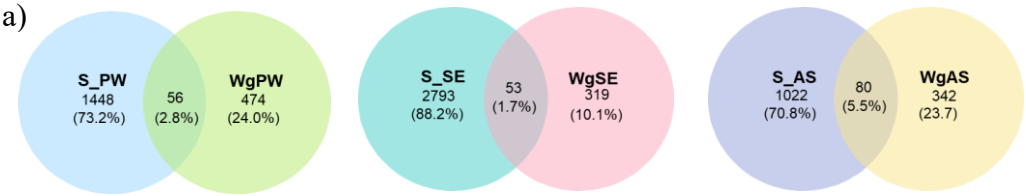


Fig. 2. 4 Venn diagram showing the percentage of recruited and unique ASVs in the *Wolffia* microbiome from its microbial sources.

Alpha diversity analysis using the Shannon and Chao1 indices revealed significant differences between sample groups (ANOVA, $p < 0.001$) (Fig. 2.5). *Wolffia* groups commonly displayed lower diversity and richness compared to their microbial sources, with significant differences (Tukey HSD test, $p < 0.05$) noted only between WgPW and S_PW for the Shannon index (Fig. 2.5a) and between WgSE and S_SE for the Chao1 index (Fig. 2.5b). These findings suggest that *Wolffia* selectively assembles specific bacterial populations from its microbial sources, resulting in reduced bacterial diversity.

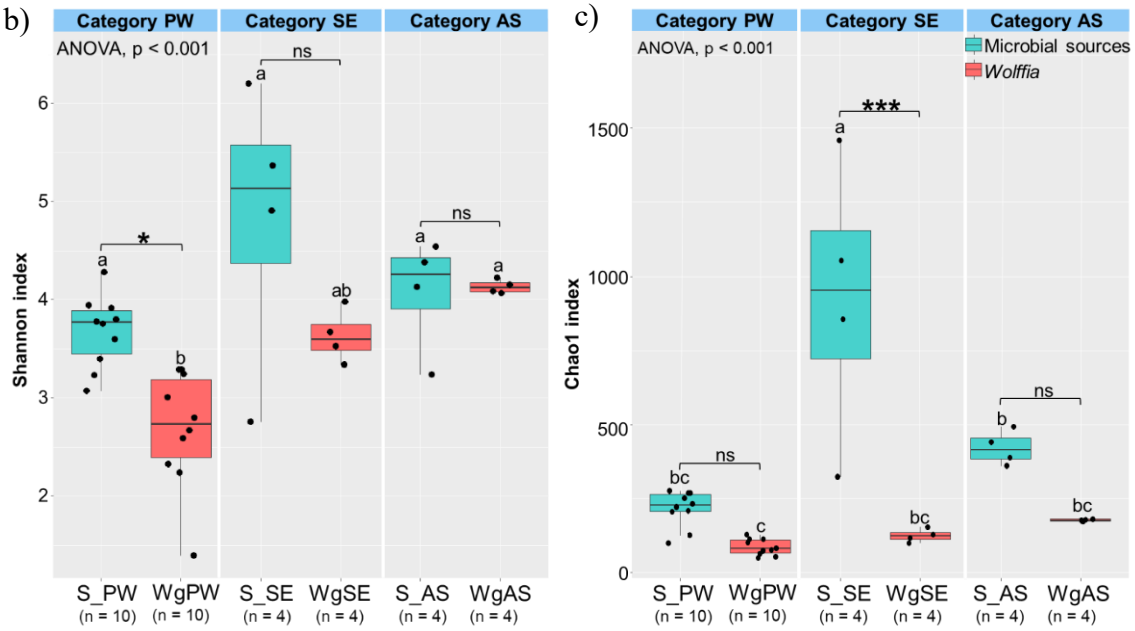


Fig. 2. 5 Alpha diversity based on b) Shannon and c) Chao1 indices. Letters indicate significant differences among groups within and across categories. Asterisks (*) denote significant differences between *Wolffia* and microbial sources within the same category (ANOVA Tukey's HSD test, *$p < 0.05$, ***$p < 0.001$, ns: no significant difference).

PCoA based on the Bray-Curtis matrix confirmed that *Wolffia* microbiomes were congregated with small separations and formed distinct clusters from microbial sources (Fig. 2.6). PERMANOVA further demonstrated that the *Wolffia* microbiome significantly differed from their microbial sources ($R^2 = 0.14145$, $p = 0.001$), and its compositional structure varied across the microbial sources ($R^2 = 0.29035$, $p = 0.001$), suggesting that *Wolffia* selectively harbors specific taxa depending on the microbial source.

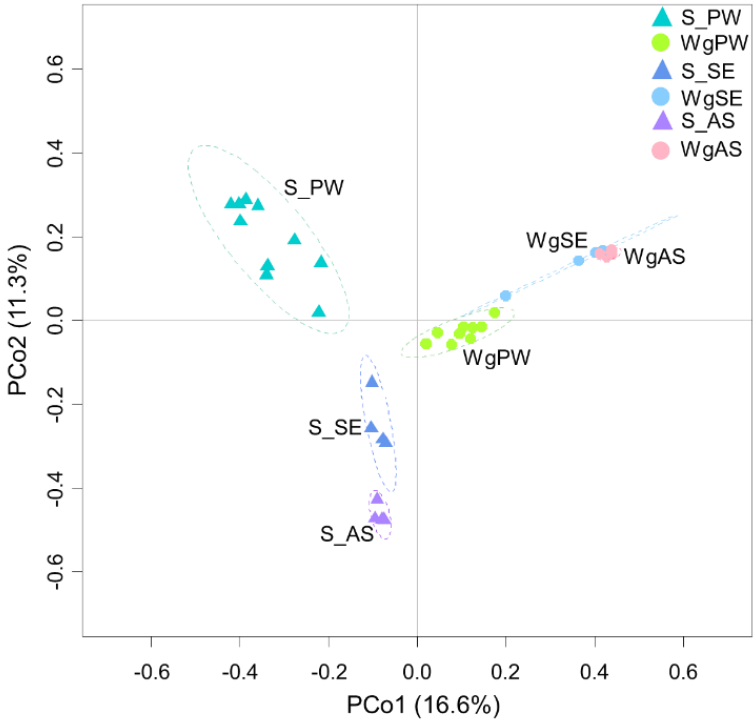


Fig. 2. 6 Principal-coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity.

The taxonomic distribution of bacterial communities highlighted notable differences between *Wolffia* microbiomes and their microbial sources across all three categories. *Wolffia* displayed reduced phyla diversity but increased abundance of specific phyla compared to the corresponding microbial sources (Fig. 2.7a). *Proteobacteria* was the most dominant phylum in microbial sources, comprising 37.9–85.3% in S_PW, 37.4–50.0% in S_SE, and 30.2–62.5% in S_AS. In contrast, *Wolffia* microbiomes had a significantly higher abundance, ranging from 83.6–99.9% in WgPW, 83.4–99.2% in WgSE, and 79.6–83.6% in WgAS. Other prevalent phyla in microbial sources included *Actinobacteriota* in S_PW (11.4–58.1%) and *Bacteroidota* in S_SE (12.2–44.9%) and S_AS (32.2–62.5%). Notably, *Actinobacteriota* was absent in *Wolffia* microbiomes, while *Planctomycetota* was uniquely dominant in WgAS (6.4–13.6%). At the class level, microbial sources mainly consisted of *Gammaproteobacteria* (23.4–82.9%) and *Actinobacteria* in S_PW (11.3–56.0%) in S_PW, and *Bacteroidia* in S_SE (12.1–44.9%) and S_AS (32.0–65.3%) (Fig. 2.7b). In *Wolffia* microbiomes, *Gammaproteobacteria* (9.6–92.4%) and *Alphaproteobacteria* (6.1–89.6%) were dominant across all categories, except in WgAS, where *Planctomycetes* was also prominent at 6.2–13.4%.

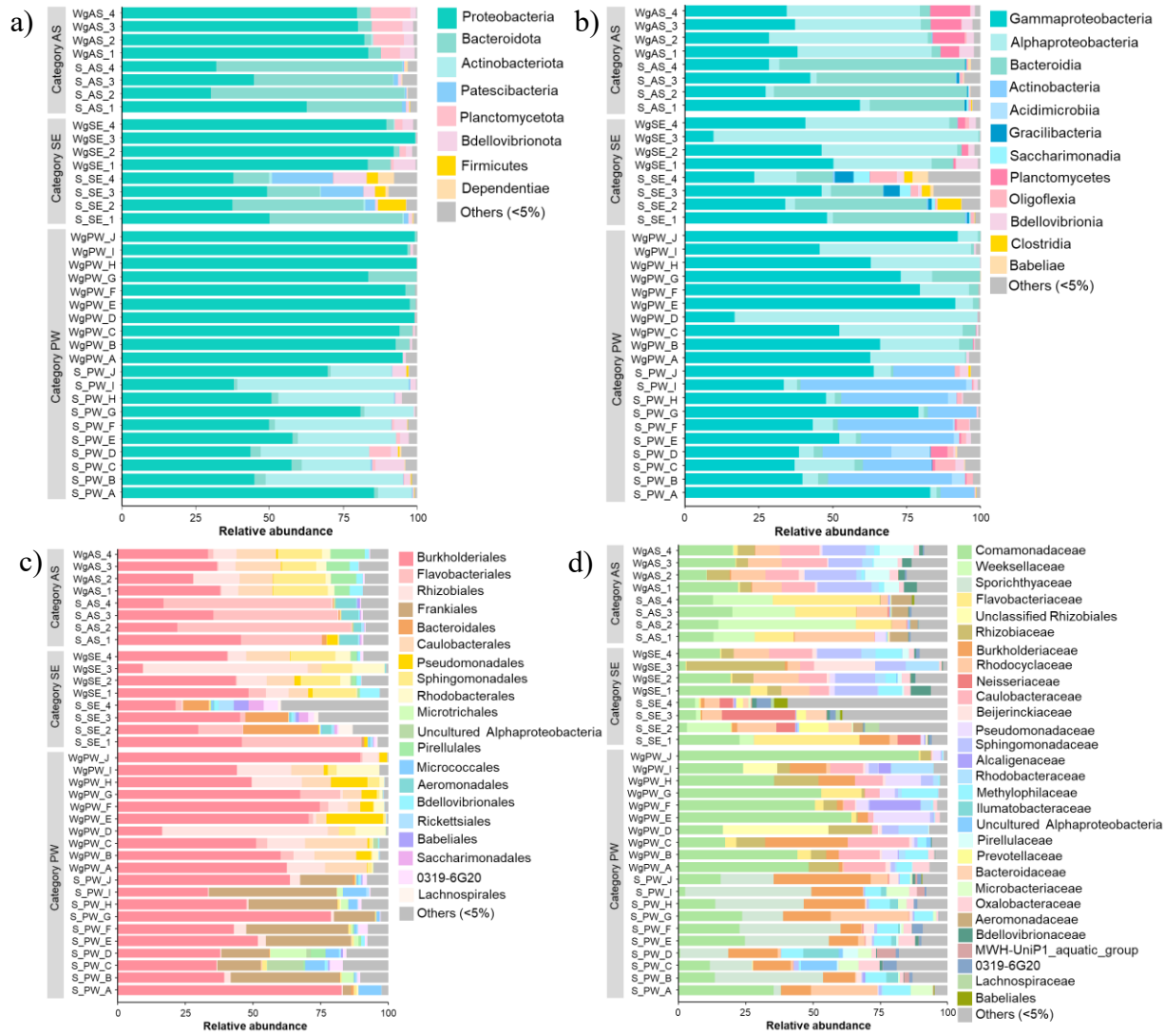


Fig. 2. 7 Taxonomic composition of bacterial communities in microbial sources and *Wolffia* microbiome at a) phylum, b) class, c) order, and d) family levels. Others include all the classified and unclassified taxa with a relative abundance of less than 5% in the sample.

At the order and family levels, differences in dominant taxa between *Wolffia* and its microbial sources became more apparent (Fig. 2.7c and Fig. 2.7d). At the family level, distinct community compositions between *Wolffia* and its microbial sources were observed, with the dominant families varying by category. In S_PW, *Sporichthyaceae* (2.8–47.1%), *Burkholderiaceae* (7.8–36.1%), and *Comamonadaceae* (0.8–35.2%) were the three predominant families, followed by *Rhodocyclaceae* (<0.1–28.9%), *Ilumatobacteraceae* (<0.1–13.3%), and *Methylophilaceae* (0.9–10.4%). In WgPW, while *Comamonadaceae* was the most dominant family (16.5–89.0%) and *Burkholderiaceae* maintained similar abundance levels (<0.1–30.7%), the other predominant families in S_PW showed a significant reduction. Instead,

Caulobacteraceae (0.4–22.8%), *Pseudomonadaceae* (<0.1–21.0%), *Rhizobiacea*e (0.4–16.5%), *Rhodobacteraceae* (<0.1–15.7%) and unclassified *Rhizobiales* (<0.1–39.1%) were predominated in WgPW. In particular, *Pseudomonadaceae* and *Rhizobiaceae* undetectable in S_PW, were exclusively enriched in significant abundance in WgPW. In category SE, *Neisseriaceae* (4.9–27.2%), *Comamonadaceae* (3.3–22.7%), *Flavobacteriaceae* (0.2–39.1%), *Weeksellaceae* (1.6–16.4%), and *Rhodocyclaceae* (2.9–14.6%) predominated in the microbial sources (S_SE). In contrast, WgSE was dominated by *Rhizobiaceae* (4.4–36.7%), *Comamonadaceae* (2.6–26.7%), *Rhodocyclaceae* (4.8–16.8%), *Sphingomonadaceae* (11.2–16.5%), and *Caulobacteraceae* (5.2–16.0%). Also, *Beijerinckiaceae* (0.2–22.7%), *Rhodobacteraceae* (0.5–12.2%) and *Methylophilaceae* (0.2–9.6%) were commonly present. Most of these families, other than *Comamonadaceae* and *Rhodocyclaceae*, were significantly enriched on *Wolffia*. In category AS, the microbial source (S_AS) was dominated by *Weeksellaceae* (15.3–51.0%), *Flavobacteriaceae* (13.2–39.8%), and *Rhodocyclaceae* (2.6–29.8%), followed by *Comamonadaceae* (12.7–20.0%) and *Aeromonadaceae* (2.9–7.4%). In contrast, *Wolffia* (WgAS) exhibited distinct composition, with *Comamonadaceae* (10.4–22.3%), *Sphingomonadaceae* (12.7–20.0%), and *Caulobacteraceae* (12.4–16.8%) as the three most dominant families. *Pirellulaceae* (5.6–12.7%), *Rhodocyclaceae* (9.0–12.7%), and *Rhizobiaceae* (4.1–8.6%) were also prevalent in WgAS.

2.3.3 Core taxa in Wolffia microbiome

The core microbiome of *Wolffia* was identified by selecting taxa with an occurrence frequency greater than 0.7 in each category, irrespective of their relative abundance. This resulted in 100 ASVs, which made up 9.3% of the total ASVs observed across all *Wolffia* samples. These ASVs accounted for 22.7%, 55.0%, and 83.4% of the relative abundance in the WgPW, WgSE, and WgAS, respectively. Although the core microbiome of each category exhibited unique ASVs, four ASVs (ASV_0006, ASV_0021, ASV_0050, ASV_0095) were shared across all categories (Fig. 2.8a). The 100 ASVs represented 7 bacterial phyla, 33 families, and 55 genera (Fig. 4b), with *Proteobacteria* being the most abundant and consistently conserved across all categories. Among the 33 families, six families—*Beijerinckiaceae, Caulobacteraceae, Comamonadaceae, Methylophilaceae, Rhizobiaceae,* and *Sphingomonadaceae*—were consistently conserved and identified as core taxa in *Wolffia* microbiome. At the genus level, 7 of the 55 genera were consistently found in high abundance across all categories, namely *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Bosea, Brevundimonas, Caulobacter, Hydrogenophaga, Methylophilus*, and *Porphyrobacter,* with

higher abundance in WgPW compared to WgSE and WgAS. Each *Wolffia* category also had specific genera that were more abundant (Fig. 2.8b), with WgSE and WgAS exhibiting similar high-abundance clusters.

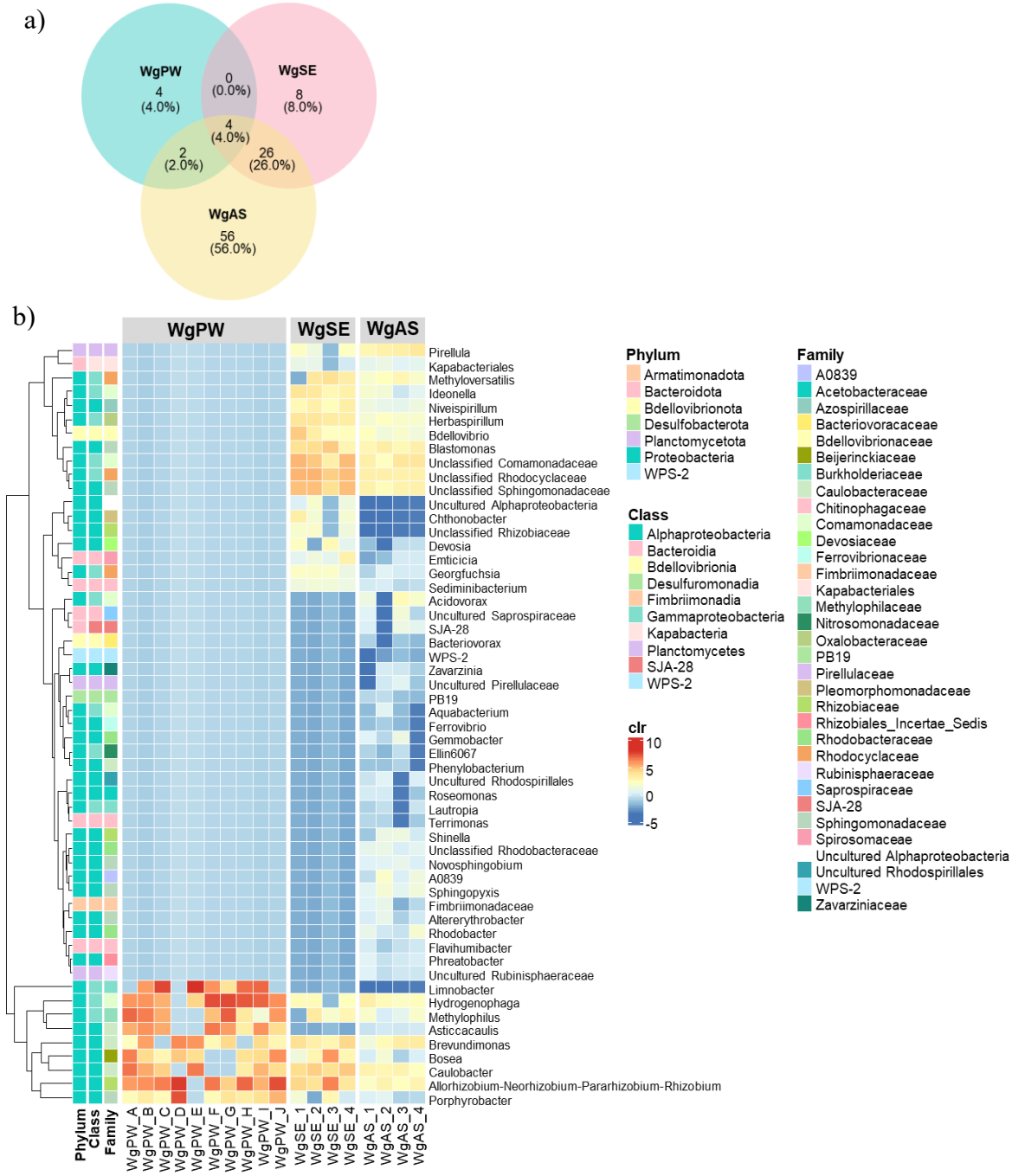


Fig. 2. 8 Core taxa of the *Wolffia* microbiome defined by ASVs with an occurrence frequency greater than 0.7 per *Wolffia* category, regardless of their relative abundance. a) Venn diagram showing shared and unique ASVs and b) heatmap presenting the 55 core genera.

2.3.4 Potential factors influencing the shaping of bacterial communities.

Given the distinct structural distribution between microbial sources and *Wolffia* microbiomes, an RDA was conducted to explore potential factors influencing community variation, including 3 environmental factors and 29 bacterial families (Table 2.2), which significantly explained 95.88% of the total variation in community structure (ANOVA, $p < 0.001$; Fig. 2.9a). The presence of bacterial families among the *Wolffia* communities (69.9%) strongly contributed to community dissimilarity, more than water quality. Concentrations of TDN and PO_4–P and the presence of *Sporichthyaceae* were the three most significant factors (Fig. 2.9b). *Burkholderiaceae* and *Ilumatobacteraceae* positively correlated with *Sporichthyaceae*, orienting S_PW toward the top left direction. Conversely, the presence of *Neisseriaceae* and *Weeksellaceae* and two environmental factors drove the community of S_SE and S_AS toward the lower-left direction.

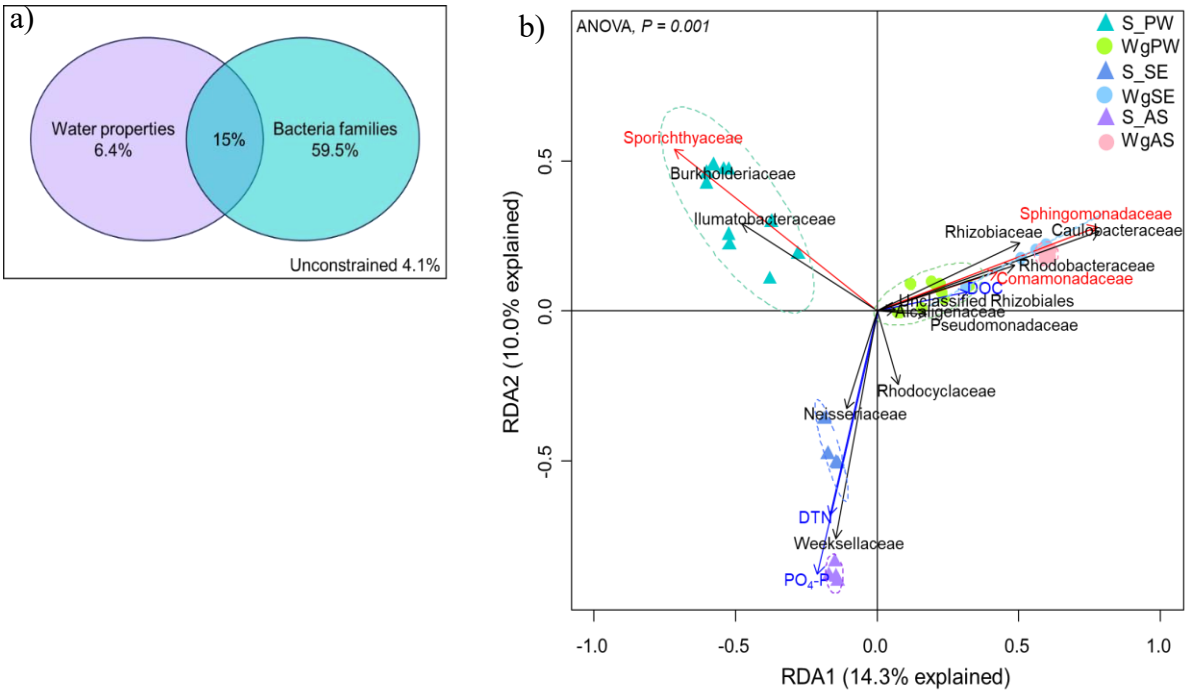


Fig. 2. 9 Potential factors driving community structure in microbiome. a) Variation partitioning analysis of bacterial diversities among the *Wolffia* and original microbial sources samples explained by water properties and bacteria families, b) redundancy analysis included statistically significant explanatory variables responded to the microbiome structure (ANOVA, $p = 0.001$). The significant factors were selected by forward selection indicated in red (bacteria families) and blue (environmental factors) colors. The length of each arrow indicates the contribution of parameters to the structural variation.

In contrast, the structure of *Wolffia* microbiomes (WgPW, WgSE, and WgAS) congregated on the right driven by DOC, and positively correlated with the presence of eight bacterial families, with *Sphingomonadaceae* and *Comamonadaceae* being the two most important factors. These findings contributed to the distinctiveness of *Wolffia* microbiome from their microbial sources, supporting the PCoA analysis.

Table 2.2 The explanatory variables included the RDA model such as three environmental factors (DOC, DTN, and PO4-P) and 29 families (relative abundance greater than 5%).

Explanatory factors	Df	Variance	F	Pr(>F)	
DOC	1	0.067272	5.4909	0.001	***
TDN	1	0.063749	5.2033	0.001	***
PO4-P	1	0.060134	4.9083	0.001	***
Comamonadaceae	1	0.037677	3.0753	0.001	***
Weeksellaceae	1	0.017722	1.4465	0.036	*
Sporichthyaceae	1	0.09782	7.9843	0.001	***
Flavobacteriaceae	1	0.016338	1.3336	0.122	
Unclassified.Rhizobiales	1	0.029199	2.3832	0.001	***
Rhizobiaceae	1	0.022912	1.8701	0.006	**
Burkholderiaceae	1	0.039946	3.2605	0.001	***
Rhodocyclaceae	1	0.031265	2.5519	0.001	***
Neisseriaceae	1	0.026822	2.1893	0.002	**
Caulobacteraceae	1	0.029605	2.4164	0.002	**
Beijerinckiaceae	1	0.018475	1.5079	0.053	
Pseudomonadaceae	1	0.023182	1.8921	0.006	**
Sphingomonadaceae	1	0.026794	2.187	0.003	**
Alcaligenaceae	1	0.023386	1.9088	0.005	**
Rhodobacteraceae	1	0.02175	1.7753	0.017	*
Methylophilaceae	1	0.018009	1.4699	0.07	
Ilumatobacteraceae	1	0.02085	1.7018	0.011	*
Pirellulaceae	1	0.013538	1.105	0.332	
Prevotellaceae	1	0.01616	1.319	0.136	
Bacteroidaceae	1	0.015979	1.3042	0.143	
Microbacteriaceae	1	0.015048	1.2283	0.204	
Oxalobacteraceae	1	0.015028	1.2266	0.203	
Uncultured.Alphaproteobacteria	1	0.012832	1.0474	0.385	
Aeromonadaceae	1	0.015005	1.2247	0.177	
Bdellovibrionaceae	1	0.013778	1.1246	0.301	
MWH.UniP1_aquatic_group	1	0.012412	1.0131	0.447	
X0319.6G20	1	0.012077	0.9858	0.479	
Lachnospiraceae	1	0.011933	0.974	0.512	
Babeliales	1	0.008956	0.731	0.859	
Residual	3	0.036755			

Significant codes: ***<0.001, **< 0.01, *< 0.05

2.3.5 Functional properties of bacterial communities

The predicted functional properties of the *Wolffia* microbiome, based on the KEGG orthologs, showed notable differences from their microbial sources, with no significant variation among *Wolffia* microbiomes across the three categories. Genes involved in motility and cellular community processes, such as bacterial chemotaxis, flagellar assembly, biofilm formation, and quorum sensing, were significantly enriched in *Wolffia* microbiomes (Fig. 2.10). Additionally, genes associated with bacterial secretion systems, ABC transporters, and two-component systems related to membrane transport and signal transduction were enriched in *Wolffia* microbiomes. Metabolic pathways linked to xenobiotic biodegradation, including amino-/benzoate degradation and glyoxylate and dicarboxylate metabolism, as well as pathways related to amino acid, energy, and carbohydrate metabolism (i.e., nitrogen/sulfur, propanoate, and butanoate metabolism), were also more abundant. Furthermore, the *Wolffia* microbiomes significantly enriched genes associated with porphyrin metabolism.

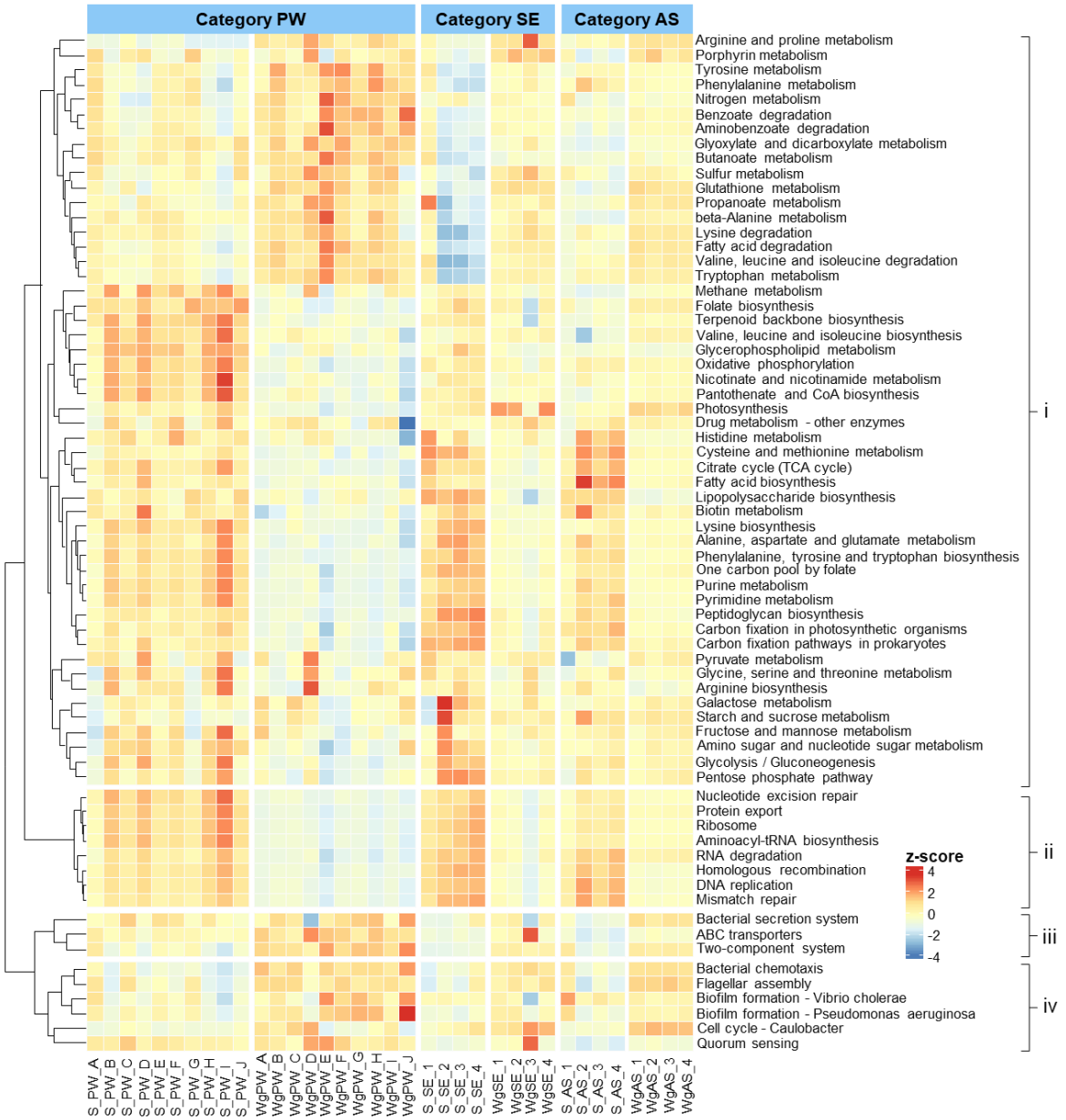


Fig. 2. 10 Functional properties of bacterial communities of *Wolffia* and microbial sources predicted by PICRUSt2 categorized into: (i) metabolism, (ii) genetic information processing, (iii) environmental information processing, and (iv) cellular processes. Relative abundance of gene categories was compared among the communities and the z-score is shown.

2.3.6 Potential key players associated with Wolffia growth

A correlation analysis examining the relationship between bacterial family abundance and *Wolffia* growth was performed to better understand the key taxa influencing *Wolffia* growth (Fig. 2.11). The analysis found a positive correlation between *Wolffia* growth and 12 bacterial families (Fig. 2.12). Notably, *Sphingomonadaceae* and *Rhodocyclaceae* were strongly associated with enhanced growth. This was consistent with taxonomic distribution and EPG results, showing their enrichment in promoting communities. Interestingly, *Bdellovibrionaceae*, a family of obligate predatory bacteria, also correlated positively with *Wolffia* growth. Conversely, seven bacterial families, including *Burkholderiaceae, Comamonadaceae*, and *Pseudomonadaceae*, were negatively associated with *Wolffia* growth due to their dominance in inhibitory communities. Although *Comamonadaceae* was present in promoting categories (WgSE and WgAS), its abundance was lower than in WgPW, suggesting that other dominant families mitigated its effects.

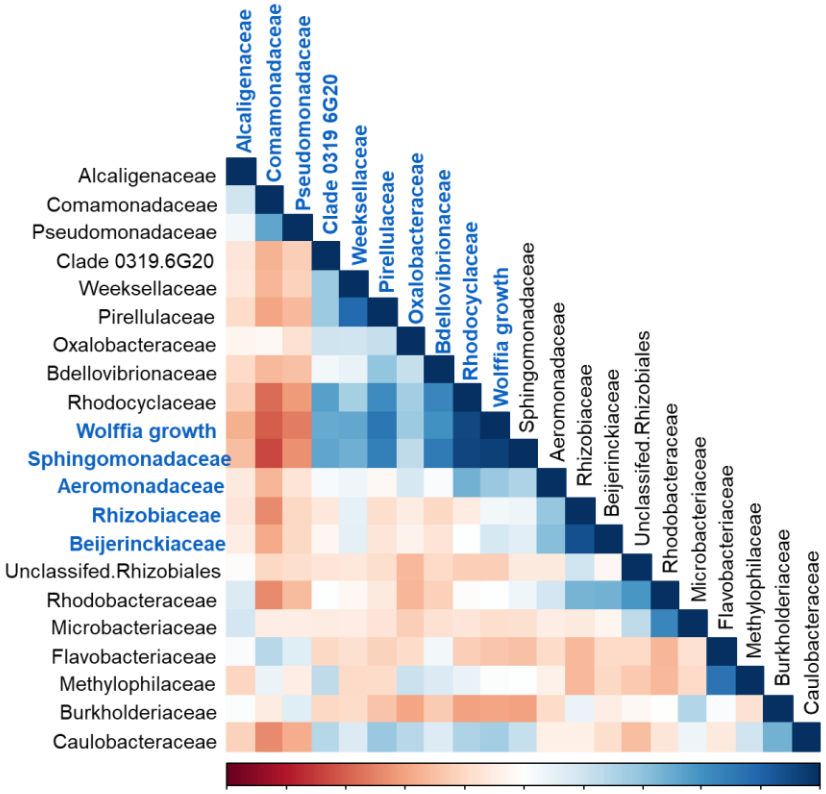


Fig. 2. 11 Hierarchical clustering of co-occurrence relationship between bacterial families and *Wolffia*'s growth. Correlations were calculated by the relative abundances of bacterial families and the EPG values for *Wolffia* growth. Blue and red indicate the positive and negative correlations, respectively. Families potentially associated with *Wolffia*'s growth are highlighted in bold blue text.

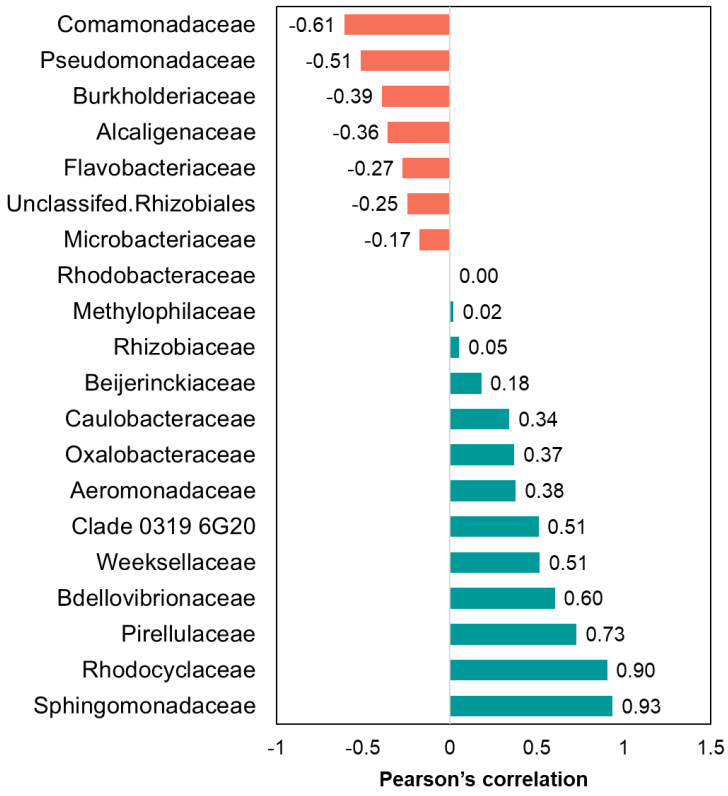


Fig. 2. 12 Positive/negative correlations between *Wolffia* growth and bacterial taxa considering as potential key families associated with *Wolffia* growth.

2.4 Discussion

This study demonstrated that bacterial communities derived from AS and SE potentially promote *Wolffia* growth (Fig. 2.1). Given that the duckweed growth is determined by the integrated activities of PGPB and PGIB in its microbiome (Glick, 2012; Ishizawa et al., 2017; Toyama et al., 2021), municipal wastewater environments may provide diverse or robust PGPB for *Wolffia*. Notably, our results suggested that PW-derived bacteria communities may not be effective for the growth of *Wolffia*. This was inconsistent with the previous findings in *Lemna minor* reporting that most of PW-derived bacterial communities exhibited promotive or neutral effects (Ishizawa et al., 2017). The presence of roots of duckweeds, which alter their interaction with bacterial communities, may cause different effects on *Wolffia* and *L. minor*, although further studies are required.

Wolffia selectively recruited and assembled bacterial communities from the inoculated microbial sources to shape its microbiome, which exhibited lower diversity compared to its microbial sources (Fig. 2.4, Fig. 2.5). This agrees with previous findings in rootless and rooted

duckweeds cultured with various microbial sources (Inoue et al., 2022a; Xie et al., 2015). Among the three categories of *Wolffia* microbiome, the PW-derived microbiome had the lowest diversity, challenging our initial expectations and contradicting the findings of Inoue et al. (2022a). Possibly, microbes in municipal wastewater environments may possess superior colonization abilities and a stronger response to plant exudates, allowing them to adhere more effectively to the *Wolffia* surface and occupy ecological niches (Ishizawa et al., 2019).

Wolffia microbiome exhibited a distinct community composition from their microbial sources because the enrichment of specific taxa (Fig. 2.6, Fig. 2.7). *Proteobacteria* comprised most of the *Wolffia* microbiome across all microbial sources, while phyla such as *Actinobacteriota* and *Bacteroidota* were abundant in the microbial sources but diminished in *Wolffia* microbiome. The dominance of *Proteobacteria* aligns with the findings in *Wolffia australiana* and rooted duckweeds (Acosta et al., 2020; Bunyoo et al., 2022; Huang et al., 2020; Inoue et al., 2022a; Xie et al., 2015). Herein, *Planctomycetota* emerged as a unique dominant phylum in *Wolffia* microbiome cultivated with AS-derived microbes. To the best of our knowledge, *Planctomycetota* has not been previously reported as a dominant phylum in duckweed microbiomes.

The dominant bacterial families in the *Wolffia* microbiome exhibited distinct patterns depending on the microbial source (Fig. 2.7d). Nevertheless, *Comamonadaceae*, *Caulobacteraceae*, and *Rhizobiaceae* were consistent dominant families. The other unique dominant families included *Burkholderiaceae* and *Pseudomonadaceae* specific to PW-derived *Wolffia* microbiome, *Rhodocyclaceae* and *Sphingomonadaceae* in municipal wastewater (SE/AS)-derived *Wolffia* microbiome, *Beijerinckiaceae* in SE-derived *Wolffia* microbiome, and *Pirellulaceae* in AS-derived *Wolffia* microbiome. Most of the dominant families identified in the *Wolffia* microbiome in this study were consistent with those found in rooted duckweeds, with the exception of *Beijerinckiaceae* (Acosta et al., 2020; Bunyoo et al., 2022; Inoue et al., 2022a): hence, these bacterial families are commonly dominant groups in duckweeds, irrespective of the presence of roots and microbial sources. Importantly, this study reflects the apparent phenomena of microbiome recruitment in duckweeds, as *Pseudomonadaceae* and *Rhizobiaceae* were enriched in the *Wolffia* microbiome, even though they were undetectable in the corresponding microbial sources. This was most evident in the PW-derived *Wolffia* microbiome. The selection of those families is likely due to the fact that they are part of the core microbiome in duckweeds (Garrido-Oter et al., 2018; Trivedi et al., 2020) and are likely skilled in adaptation to the plant environment, leading to their preferential colonization and

dominance in *Wolffia* microbiome, with complicated interactions in microbial networks, regardless of their abundance in surrounding environments (Agler et al., 2016).

Despite the distinct dominant families across different microbial sources, *Beijerinckiaceae, Caulobacteraceae, Comamonadaceae, Methylophilaceae, Rhizobiaceae,* and *Sphingomonadaceae* were consistently conserved and identified as core taxa in the *Wolffia* microbiome (Fig. 2.8). These core families generally resemble those commonly found in the rooted duckweed microbiome (Acosta et al., 2020; Bunyoo et al., 2022; Huang et al., 2020; Inoue et al., 2022a). Exceptionally, *Beijerinckiaceae* was uniquely identified as a core taxon in *Wolffia* microbiome, which are discussed below in details. These taxa belong to *Proteobacteria*, a phylum commonly associated with plant microbiome, where it exhibits symbiotic, harmful, or neutral relationships with the host (Maheshwari and Sankar, 2023). Additionally, as the genera, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Bosea, Brevundimonas, Caulobacter, Hydrogenophaga, Methylophilus,* and *Porphyrobacter* were consistently found in the *Wolffia* microbiome, suggesting their role as "stable" core taxa. The first three genera were identified as stable core taxa in natural *Wolffia* and shared with other duckweeds. Specifically, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* may serve as a strongly conserved core taxon, as it is consistently maintained in *Wolffia* even under nutrient-deficient conditions (Bunyoo et al., 2022). Meanwhile, *Bosea, Brevundimonas, Caulobacter,* and *Methylophilus* were newly reported here as stable core taxa in *Wolffia*, suggesting that it has unique core taxa and expanding our understanding of its core microbiome. The findings reflect that microbial community assemblage follows structural principles rather than occurring randomly or varying across microbial sources (Li et al., 2021; Vorholt, 2012). This stability indicates that microbiome assemblage is rather a deterministic process that is independent of the initial microbial abundance in the source microbiome. The uniqueness of the *Wolffia* core microbiome is likely due to niche differences between *Wolffia* and rooted duckweed, which may contribute to selective filtering and recruitment, shaping resource availability and promoting the establishment of specific taxa. Given that niche ecology is essential in shaping microbial communities (Hamonts et al., 2018; Lai et al., 2023; Leach et al., 2017; Trivedi et al., 2020), it may determine the colonization of specific microorganisms in the *Wolffia* microbiome. This phenomenon, previously observed in terrestrial plants (Lai et al., 2023), may also occur in duckweed. Xie et al. (2015) demonstrated that bacteria primarily colonized reproductive pockets (where daughter and mother fronds attach) and stomata in *W. australiana*, whereas bacteria densely aggregated beneath fronds, around roots, and at the root base in rooted

duckweed (Ishizawa et al., 2019). Furthermore, differences in exudate secretion driven by distinct morphology may cause unique plant-microbe interactions, shaping a distinct microbiome (Mercier and Lindow, 2000; Yurgel et al., 2022). These support the idea that the distinct morphology and physiology of rootless duckweed to those of rooted duckweed contribute to the formation of a unique core microbiome. However, this study defined the core microbiome based on the occurrence frequency of ASVs in each microbial source category, and the sample size in categories SE and AS was limited. Expanding the sample size is required to further validate the core microbiome in rootless duckweed.

The discrepancy in community structure between microbial sources and *Wolffia* microbiome is influenced by several factors, including fluctuations in the surrounding environment and interactions within different microbial communities (Ishizawa et al., 2024; Vorholt, 2012). The RDA found that while the communities of microbial sources are diverse, the *Wolffia* microbiomes are more clustered (Fig. 2.9). The presence of specific bacterial families such as *Sphingomonadaceae* and *Comamonadaceae* and the DOC concentration appear to be key determinants in shaping the *Wolffia* microbiome and differentiating it from microbial sources. These families were consistently enriched in *Wolffia* microbiome, indicating their significant influence on community structure. Additionally, shifts in DOC concentrations, representing the abundance of carbon sources, alter the bacterial community structure, affecting dominant taxa, diversity, and functional potential. Easily degradable DOC supports diverse bacterial populations compared to more complex or recalcitrant organic matter (Ávila et al., 2019; Chen et al., 2023; Zhang and Wang, 2022; Zhou et al., 2021).

Functional profiling revealed the enrichment of genes associated with flagella assembly, biofilm formation, bacterial chemotaxis, two-component systems, and bacterial secretion systems in the *Wolffia* microbiome (Fig. 2.10). These genes support bacterial movement toward the duckweed surface in response to secreted exudates and facilitate bacterial attachment (Scharf et al., 2016), likely being crucial in the microbiomes of rooted and rootless duckweeds (Bunyoo et al., 2022; Inoue et al., 2022a; Ishizawa et al., 2020a). Notably, quorum sensing (QS) and ABC transporters were specifically enriched in the *Wolffia* microbiome. QS may serve as a unique functional property supporting microbiome assembly on *Wolffia*. Given that *Wolffia* lacks roots to host bacterial communities, QS likely enables bacteria to communicate and synchronize their behavior to effectively colonize *Wolffia* surfaces. The exudates secreted by rootless duckweed may act as signaling molecules, triggering bacterial QS communication and influencing plant-microbe interactions. QS signals regulate biofilm formation in a density-

dependent manner, promoting bacterial aggregation and colonization on plant surfaces (Hartmann et al., 2014; Majdura et al., 2023; Williams, 2007). In turn, they modulate plant growth and defense mechanisms by fostering beneficial bacterial interactions and allowing the bacteria to thrive in a symbiotic relationship (Schikora et al., 2016; Shrestha et al., 2020). ABC transporters contribute to nutrient uptake, enabling bacteria to acquire essential molecules, including sugars, amino acids, and specific plant metabolites required for their survival and colonization within the host plant microbiome (Davidson et al., 2008). Some ABC transporters may be involved in sensing plant signals, allowing bacteria to adapt their behavior in response to the plant environment, facilitating beneficial plant-microbe interactions. These functional properties facilitate bacterial colonization, provide a competitive advantage of dominant taxa, and enhance bacterial adaptation. Most genes related to amino acid and carbon metabolisms were also significantly enriched in *Wolffia* microbiome. This may be due to the *Wolffia* microbiome response to utilize metabolites secreted by *Wolffia*.

Correlation analysis revealed that *Wolffia* growth is influenced by the increase of specific dominant families (Fig. 2.11, Fig. 2.12). Particularly, the increase of *Spingomonadaceae* and *Rhodocyclaceae* had a strong positive relationship with *Wolffia* growth. This is consistent with their significant enrichment in the promoting community (WgSE and WgAS). In addition to being a key player in *Wolffia* growth, *Sphingomonadaceae* is one of the core taxa and a key factor in shaping the *Wolffia* microbiome. Members of this family are commonly found in the terrestrial plant phyllosphere and contribute to plant growth promotion, protection, stress resilience, and competitiveness (Innerebner et al., 2011; Kaczmarczyk et al., 2011; Vogel et al., 2012). In addition to *Sphingomonadaceae* and *Rhodocyclaceae*, *Beijerinckiaceae* was not only a unique core taxon in the *Wolffia* microbiome but also influenced *Wolffia* growth and was enriched in WgSE. To the best of our knowledge, this is the first report identifying *Beijerinckiaceae* as a keystone taxon in the duckweed microbiome. *Beijerinckiaceae* is uncommon in rooted duckweed microbiome; however, it is commonly found in plant rhizosphere and phyllosphere and aquatic environments (Marín and Arahal, 2014; Yuan et al., 2021). Members of this family are recognized as versatile bacterial groups that perform methanotrophy, methylotrophy, nitrogen fixation, and phosphate mobilization, while being essential within the phyllosphere. Additionally, they can establish their populations, outcompete other microbes, and contribute to microbial community dynamics (Camargo et al., 2023; Duan et al., 2024). Furthermore, *Beijerinckiaceae* secrete plant regulators, amino acids, and nitrogenous compounds assimilated by plants. In turn, plant exudates provide nutrients that

microbes can utilize (Miyasaka et al., 2003; Polyanskaya et al., 2002; Thuler et al., 2003), potentially facilitating a specific interaction between *Beijerinckiaceae* and *Wolffia*.

Another interesting aspect is that *Bdellovibrionaceae* had a positive relationship with *Wolffia* growth (Fig. 2.12). Inoue et al. (2022a) also found highly enrichment of *Bdellovibrionaceae* specifically in rootless duckweed microbiome. *Bdellovibrionaceae* is known as a group of obligate predatory bacteria that actively prey on gram-negative bacteria and has not been reported as PGPB. Obligate predatory bacteria such as *Bdellovibrionaceae* do not directly affect plant growth or compete for nutrients with heterotrophic bacteria. However, in microbial ecosystems, they contribute to shaping microbiome structure through predator-prey interactions, which in turn influence plant health (Guo et al., 2022; Inoue et al., 2022b). The evidence suggests that *Bdellovibrionaceae* is crucial as the population balancer/controller in the rootless duckweed microbiome (Inoue et al., 2023).

Overall, our results suggest that *Bdellovibrionaceae, Beijerinckiaceae*, and *Sphingomonadaceae* may serve as "hub microorganisms" and "keystone taxa" in the *Wolffia* microbiome. These families represent core taxa, act as mediators, and strongly interact with the plant and microbes, influencing microbiome structure and plant growth; this allows them to firmly integrate into the *Wolffia* holobiont, retaining their role for an extended period. These findings suggest that members of these families may function as potential PGPB for *Wolffia* in a broad sense that can enhance its productivity directly or indirectly. To confirm this hypothesis, further studies are needed to isolate strains of the potential taxa listed in this study and clarify their plant growth-promoting activities. In addition, while this study successfully investigated the effects of diverse bacterial communities and presented the *Wolffia* microbiome, the bacterial abundance in the original microbial sources was not normalized, which might have influenced the promotion/inhibition of *Wolffia* growth. While the main microbial groups and community organization likely remain consistent, how bacterial abundance influences *Wolffia* growth and associated microorganisms represents a critical question for subsequent research. Furthermore, our correlation analysis suggested that the estimated positive and negative correlations (Fig. 2.12) stem not only from individual bacterial families but also from interactions between multiple groups (Fig. 2.11). The findings emphasize the need to clarify bacterial interactions for better understanding the functions of the *Wollfia* microbiome as a system.

2.5 Summary

In this chapter, co-cultivation of *W. globosa* with bacterial communities derived from freshwater ponds, municipal secondary effluent, and activated sludge was conducted to systematically characterize the *Wolffia* microbiome, including the phylogenetic composition and effect on *W. globosa* growth. Municipal wastewater-derived bacteria communities had a more pronounced positive effect on *W. globosa* growth compared with those from pond water. Although the composition of the *Wolffia* microbiome varied depending on the microbial sources, six bacterial families were identified as core taxa in the *Wolffia* microbiome: *Beijerinckiaceae, Caulobacteraceae, Comamonadaceae, Methylophilaceae, Rhizobiaceae*, and *Sphingomonadaceae*. Functional profiling of *Wolffia* microbiome also demonstrated a common enrichment of several functions associated with bacterial colonization and adaptation to the rootless morphology, contributing to selective microbiome recruitment. Redundancy and correlation analyses further elucidated that the structure of *Wolffia* microbiome is driven by DOC level and positively correlated with the presence of eight bacterial families, with *Sphingomonadaceae* and *Comamonadaceae* being the two most important factors. Furthermore, *Bdellovibrionaceae*, *Beijerinckiaceae*, and *Sphingomonadaceae* were identified as "hub microorganisms" and "keystone taxa," shaping community structure and directly or indirectly influencing *Wolffia* growth. The overall findings obtained in this chapter can be summarized as Fig. 2.13.

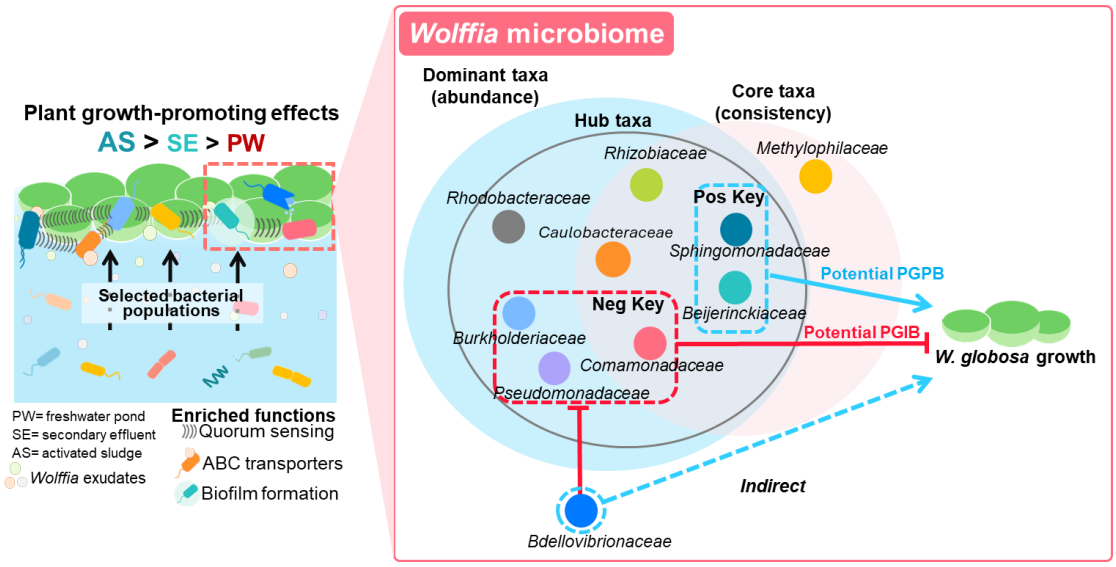


Fig. 2. 13 Summary of important characteristics of *Wolffia* microbiome.

Chapter 3 Characterization of duckweed-associated bacteria from the *Wolffia* microbiome: strain-level effects on host growth and key determinants

3.1 Introduction

Systematic characterization of the structure and functions of *Wolffia* microbiome in Chapter 2 proves that the growth of *Wolffia* is significantly influenced by the associated microbiome, ranging from growth-inhibiting to strongly growth-promoting. These contrasting phenotypes are likely the results of interactions between *Wolffia* and diverse DABs within its microbiome, including PGPB and PGIB. PGPB can improve duckweed growth, health, and phytoremediation performance, as well as protect plants from biotic and abiotic stress through multiple mechanisms such as phytohormone modulation, nitrogen fixation, phosphate solubilization, and siderophore-mediated iron supply. Conversely, PGIB can suppress duckweed growth without visible pathogenic symptoms through competition for nutrients and other unidentified mechanisms. As listed in Table 1.2 in Chapter 1, DABs have been extensively isolated from microbiomes of rooted duckweeds and characterized their properties associated with the host plant growth. However, DABs of *Wolffia* have rarely been isolated and characterized their individual effect on the host plant. Therefore, this chapter aimed to evaluate the effects of nearly 100 *Wolffia* DABs, which were isolated from *W. globosa* microbiomes established in Chapter 2, on host plant growth and comprehensively characterize their physiological traits that may be associated with the effect on host growth.

3.2 Material and Methods

3.2.1 Isolation of duckweed-associated bacteria

DABs tested in this chapter were isolated from 7 of the 18 *Wolffia* microbiomes established in Chapter 2: 4 (PW_A, PW_B, PW_F, and PW_H), 1 (SE_2), and 2 (AS_1 and AS_2) DABs originated from co-cultivation with bacterial communities derived from freshwater ponds, secondary effluents, and activated sludge, respectively. Among them, microbiomes PW_A, SE_2, AS_1, and AS_2 promoted *Wolffia* growth. Conversely, microbiome PW_B and microbiomes PW_F and PW_H had neutral and inhibitory effects, respectively (Fig. 2.1). To isolate DABs, the plants collected after 10 days of co-cultivation in the Chapter 1 were placed in sterile microtubes containing 200 µl of MH medium and homogenized using a BioMasher II (Nippi, Tokyo, Japan). The homogenates were serially

diluted, and 100 µl of each dilution was spread onto R2A agar (DAIGO, Shiotani M.S. Co., Ltd., Japan). The plates were incubated at 28 °C for 3–7 days. All morphologically distinct colonies from each sample were isolated and purified on the same medium. The isolates were routinely maintained on the same medium and stored in R2A broth containing 25% glycerol at -80 °C for long-term preservation. Unless otherwise noted, all cultivations were performed at 28 °C, with rotary shaking at 120 rpm in liquid medium.

3.3.2 Taxonomic identification

Genomic DNA of isolated bacteria was extracted using the Cica Geneus DNA extraction reagent (Kanto Chemical, Japan). The genomic DNA was amplified to obtain partial sequences of the 16S rRNA gene using SapphireAmp Fast PCR Master Mix (Takara Bio Inc., Japan) and universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, CA, USA). PCR amplification was performed with the following thermocycling conditions: initial denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were purified via a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and sequenced using an Applied Biosystems 3730xl DNA analyzer (Macrogen Japan Corp., Japan). The obtained 16S rRNA gene sequences were compared with type strain sequences in the EzBioCloud database (https://www.ezbiocloud.net/) (Yoon et al., 2017) for taxonomic identification. A phylogenetic tree was constructed using the neighbor-joining method alongside the maximum composite likelihood model in MEGA 11 (Tamura et al., 2021). The 16S rRNA gene sequences of the isolated DABs were deposited in the GenBank/EMBL/DDBJ databases under accession numbers LC896431–LC896528.

3.2.3 Co-cultivation experiment to assess the effects on Wolffia growth

Prior to the experiments, a loopful of each isolated strain grown on R2A agar was inoculated into a glass vial containing 20 mL of R2A broth and cultured for 24 h. Bacterial cells were harvested by centrifugation (10,000×*g*, 4 °C, 10 min), washed twice with MH medium, and then re-suspended in the same medium to obtain an optical density at 600 nm (OD_{600}) of 0.1, measured using a UV-Vis spectrophotometer (UV-1850, Shimadzu, Kyoto, Japan).

Meanwhile, axenic *W. globosa* was pre-cultivated in MH medium for 5 days under light conditions with an irradiance of 80 µmol/m^2/s and a photoperiod of 16 h light/8 h dark. Plant sterility was routinely confirmed by the absence of microbial growth on R2A agar after plating *W. globosa* with and without homogenization.

The effect of isolated strains on *Wolffia* growth was evaluated using a two-step co-cultivation experiment applied in Chapter 2 (section 2.2.2). Briefly, twenty fronds of *W. globosa* were transferred to a 6-well plate containing 10 mL of the bacterial inoculum suspension to initiate the co-cultivation experiments. The co-culture was maintained under the aforementioned light conditions for 3 days (colonization stage) to facilitate bacterial attachment to the plant. Subsequently, the entire plant was transferred to a new 6-well plate containing 10 mL of fresh bacteria-free medium and cultivated under identical conditions for 3 days (growing stage) to promote biomass growth. A control experiment was conducted using axenic *W. globosa* cultivated in sterile MH medium devoid of bacterial inoculum. Co-cultivation experiments involving each isolate, along with control experiments, were performed in quintuplicate. On day 0, 3, and 6, the frond area was measured, and the EPG value was determined to assess the impact of isolated bacteria on plant growth, as described in Chapter 2 (section 2.2.3).

3.2.4 Physiological characterization

As representative physiological characteristics that are well known to be associated with plant growth promotion, the abilities to produce siderophores, indole-related compounds (IRCs), and hydrogen cyanide (HCN) and solubilize phosphate were assessed for all isolated strains. The cells from isolated strains, precultured as described above, were re-suspended in R2A broth to achieve an OD_{600} of 1. Subsequently, 10 µl of the cell suspension was spot-inoculated onto each assay medium.

Siderophore production was evaluated on chrome azurol S (CAS) agar medium for 7 days; a change in the halo zone surrounding the colony from blue to yellow indicated positive activity. IRC production was assessed using colorimetric detection (Gilbert et al. 2018), with minor modifications. Briefly, the assay was performed in R2A broth supplemented with 0.05% (w/v) L-tryptophan for 5 days in the dark. Subsequently, the culture was centrifuged (10,000×*g*, 4 °C, 10 min), and 100 µl of the supernatant was mixed with 200 µl of Salkowski reagent. Following a 30-min incubation at room temperature, with air conditioning maintained at 25 °C and in the dark, the absorbance at 530 nm was measured to determine IRC concentrations using indole-3-acetic acid (IAA) as the standard reference. A full-spectrum scan (440–600 nm, 1-nm

intervals) was conducted to determine the maximum absorbance and classify specific IRCs based on the specificity of the Salkowski reagent as outlined by Gilbert et al. (2018). HCN production was assayed using a picrate strip for 1–2 days according to the methodology described by Saber et al. (2015). The phosphate solubilization activity was evaluated using Pikovskaya's agar medium for 7 days. Positive activity was observed in a halo zone below or around the colonies.

3.2.5 Data and statistical analyses

To validate the consistency between culturable DABs and the *Wolffia* core microbiome, the 16S rRNA gene sequences of isolated DABs were compared with those of ASVs of the core *Wolffia* microbiome (BioProject accession number PRJDB18855) using pairwise distance analysis in MEGA11. Strains exhibiting >99% similarity with core ASVs were deemed reliable representatives of core taxa within the *Wolffia* microbiome at the genus level (Zhou et al., 2024). A multi-way ANOVA was performed in R (version 4.3.2) to assess the correlation between the capacities of the isolated strains to produce siderophores, IRCs, and HCN, and solubilize phosphate with the growth promotion or inhibition of *W. globosa*, following the parameters as described by Ishizawa et al. (2017). Statistical significance was set at $p < 0.05$.

3.3 Results and discussion

3.3.1 Isolation and identification of Wolffia DABs

A total of 98 bacterial strains were isolated from *W. globosa* microbiomes after 10 days of co-cultivation with 7 distinct microbial sources. The number of strains isolated from each microbiome is summarized in Table 3.1. Of these, 70 strains were isolated from four growth-promoting microbiomes (PW_A, SE_2, AS_1, and AS_2), 9 strains from a neutral microbiome (PW_B), and 19 strains from two growth-inhibiting microbiomes (PW_F and PW_H).

Table 3. 1 Number of strains obtained from each microbiome.

Communities	Number of strains	Community level effect*	Community diversity (Shannon index)	Sources of the derived *Wolffia* microbiome
PW_A	17	Promotion	3.0	Freshwater pond
SE_2	10	Promotion	4.0	Activated sludge
AS_1	22	Promotion	4.2	Activated sludge
AS_2	21	Promotion	4.2	Activated sludge
PW_B	9	Neutrality	3.0	Freshwater pond
PW_F	6	Inhibition	2.6	Freshwater pond
PW_H	13	Inhibition	2.7	Freshwater pond

*The effect of the overall microbiome on *W. globosa* growth derived from Chapter 2.

Sequencing of the 16S rRNA gene categorized the 98 DAB strains into 30 genera across 18 families within three phyla: Pseudomonadota (formerly Proteobacteria), Bacillota, and Actinomycetota (Fig. 3.1). Most of the strains belonged to the phylum Pseudomonadota (96 strains), which comprised 16 families, mainly *Boseaceae* (or *Beijerinckiaceae* in the SILVA SSU r138.2 database), *Rhizobiaceae, Pseudomonadaceae, Methylobacteriaceae* (or *Beijerinckiaceae* in the SILVA SSU r138.2 database), and *Sphingomonadaceae*. The remaining two isolates were assigned to Bacillota (family *Staphylococcaceae*, 1 isolate) and Actinomycetota (*Mycobacteriaceae*, 1 isolate).

The isolates from the 4 growth-promoting microbiomes were dominated by *Boseaceae* (*Bosea*, 26 isolates), *Rhizobiaceae* (*Rhizobium*, 6 isolates; *Shinella*, 2 isolates; *Agrobacterium*, 2 isolates; *Ensifer*, 1 isolate), and *Sphingomonadaceae* (*Novosphingobium*, 3 isolates; *Sphingomonas*, 2 isolates; *Sphingopyxis*, 5 isolates). Meanwhile, the isolates from the 2 growth-inhibitory microbiomes predominantly belonged to the families *Pseudomonadaceae* (*Metapseudomonas*, 6 isolates; *Pseudomonas*, 1 isolate; *Aquipseudomonas*, 1 isolate) and *Rhizobiaceae* (*Rhizobium*, 4 isolates). The DABs isolated from *Wolffia* microbiomes exhibited substantial taxonomic diversity, predominantly comprising Pseudomonadota, repeatedly identified as the primary associate of duckweed, including *Wolffia* (Acosta et al., 2020; Boonmak et al., 2023; Bunyoo et al., 2022; Inoue et al., 2022a; Saimee et al., 2025). Most families found in the two primary microbiome patterns, namely growth-promoting and growth-inhibiting microbiomes, aligned with the dominant families found in Chapter 2. *Boseaceae*, *Rhizobiaceae*, and *Sphingomonadaceae* were highly dominant in the secondary effluent/activated sludge-derived *Wolffia* microbiomes (typically growth-promoting). Conversely, *Pseudomonadaceae* was exclusively dominant in the pond water-derived microbiomes (typically growth-inhibiting). These findings demonstrate consistency between culture-dependent and culture-independent data in the *Wolffia* microbiome study, indicating the successful colonization of surrounding bacterial communities on *Wolffia* and supporting the selective recruitment of these communities from the surrounding environment by *Wolffia*.

Among the 98 isolates, 54 shared more than 99% similarity in the 16S rRNA gene sequences with 22 core ASVs identified in Chapter 2 (Fig. 3.1). These matched strains belonged to 10 genera in the families *Boseaceae*, *Rhizobiaceae*, *Caulobacteraceae*, *Sphingomonadaceae*, *Oxalobacteraceae*, and *Methylophilaceae*. Collectively, these findings suggest that we successfully isolated DABs representing core taxa from the *Wolffia* microbiome.

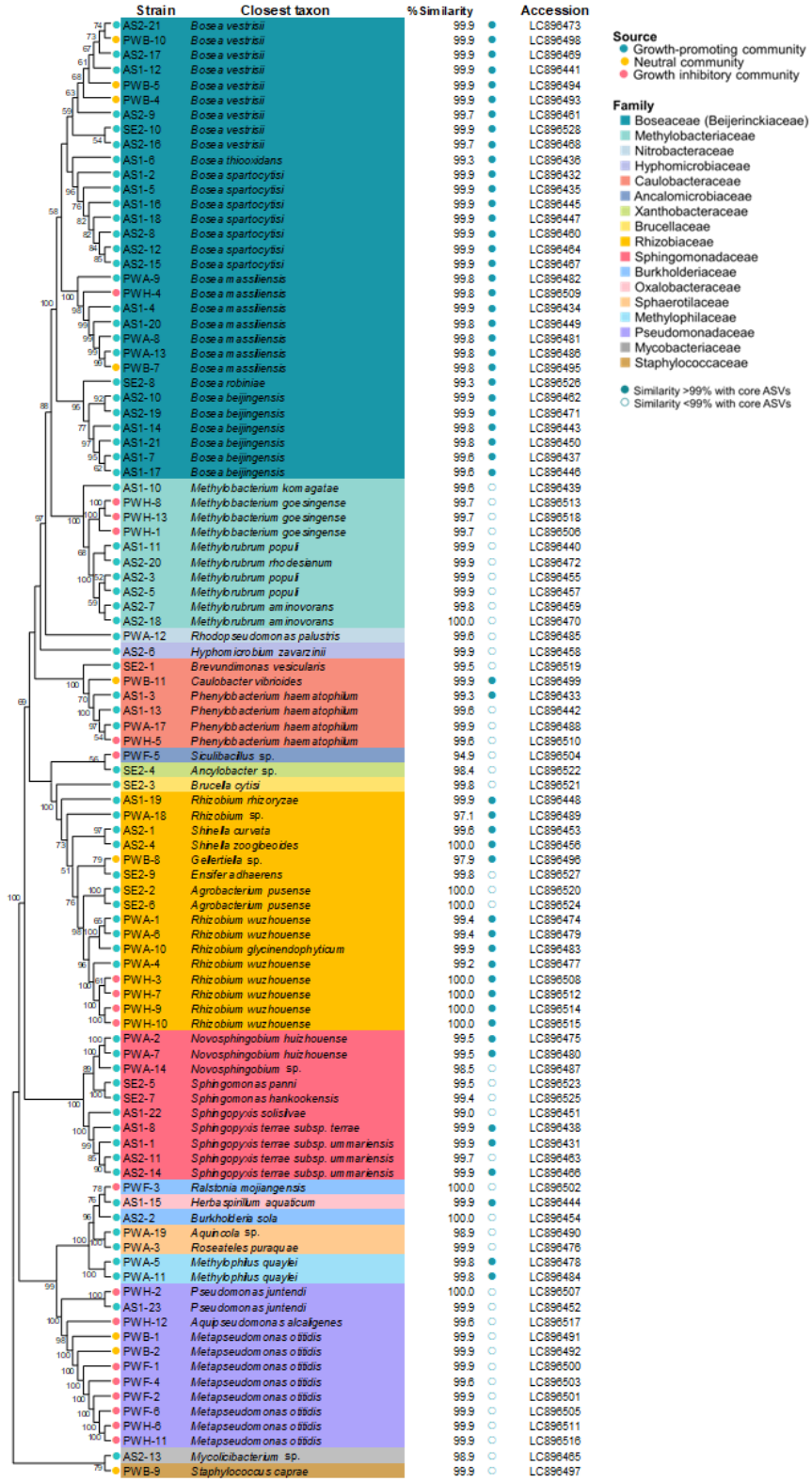


Fig. 3. 1 Phylogenetic tree based on 16S rRNA gene sequences of 98 DABs isolated from 7 *W. globosa* microbiomes in this study and their sequence similarities to the closest taxon, as well as their correspondence to ASVs from the original microbiomes (Bioproject PRJDB18855).

3.3.2 Effects of isolated DABs on Wolffia growth

Evaluation at the strain level

The 98 DAB strains demonstrated a wide range of effects on *Wolffia* growth, ranging from inhibition to promotion (Tukey post hoc test; $p < 0.05$). The EPG ranged from –28.1% to +27.8% after 3 days and from –14.6% to +12.9% after 6 days (Fig. 3.2; Fig. 3.3; Fig. 3.4). Ten strains consistently promoted *W. globosa* growth at both time points (3 and 6 days): SE2-3 (*Brucella cytisi*, 12.9 ± 1.9%), AS1-7 (*Bosea beijingensis*, 12.8 ± 3.5%), AS1-5 (*Bosea spartocytisi*, 12.7 ± 3.6%), AS1-18 (*B. spartocytisi*, 12.3 ± 3.5%), AS2-1 (*Shinella curvata*, 11.2 ± 1.9%), SE2-2 (*Agrobacterium pusense*, 11.1 ± 2.2%), AS1-14 (*B. beijingensis*, 10.7 ± 4.3%), AS1-15 (*Herbaspirillum aquaticum*, 10.6 ± 4.1%), AS1-17 (*B. beijingensis*, 10.3 ± 3.4%), and AS2-5 (*Methylorubrum populi*, 6.0 ± 3.2%). Seven PGPB strains were aligned with the core ASVs (core PGPB strains), highlighting their ecological importance in the *Wolffia* microbiome (Table 3.2).

Conversely, nine strains persistently inhibited *Wolffia* growth at both 3 and 6 days and were characterized as PGIB: PWA-7 (*Novosphingobium huizhouense*, –14.6 ± 0.8%), PWA-11 (*Methylophilus quaylei*, –11.6 ± 2.1%), AS2-2 (*Burkholderia sola*, –10.2 ± 2.2%), PWA-17 (*Phenylobacterium haematophilum*, –8.9 ± 0.8%), PWA-5 (*Me. quaylei*, –8.3 ± 1.5%), SE2-1 (*Brevundimonas vesicularis*, –6.8 ± 2.9%), PWF-3 (*Ralstonia mojiangensis*, –8.6 ± 2.3%), PWH-8 (*Methylobacterium goesingense*, –7.3 ± 1.2%), and PWA-2 (*N. huizhouense*, –6.7 ± 1.5%). Four PGIB, specifically *Me. quaylei* (PWA-5 and PWA-11) and *N. huizhouense* (PWA-7 and PWA-2), corresponded with the core ASVs (core PGIB strains), thereby experimentally confirming their role as “negative key players” detrimental to *Wolffia* productivity (Table 3.2).

Collectively, these results confirm that *Wolffia* hosts both PGPB and PGIB, a finding also noted in rooted duckweed microbiomes (Ishizawa et al., 2017). Strong and consistent effects on host plant growth were predominantly observed in a small subset of DABs (19 out of 98 DABs), with most strains displaying weak or context-dependent influences, thereby indicating complex, strain-specific host-microbe dynamics.

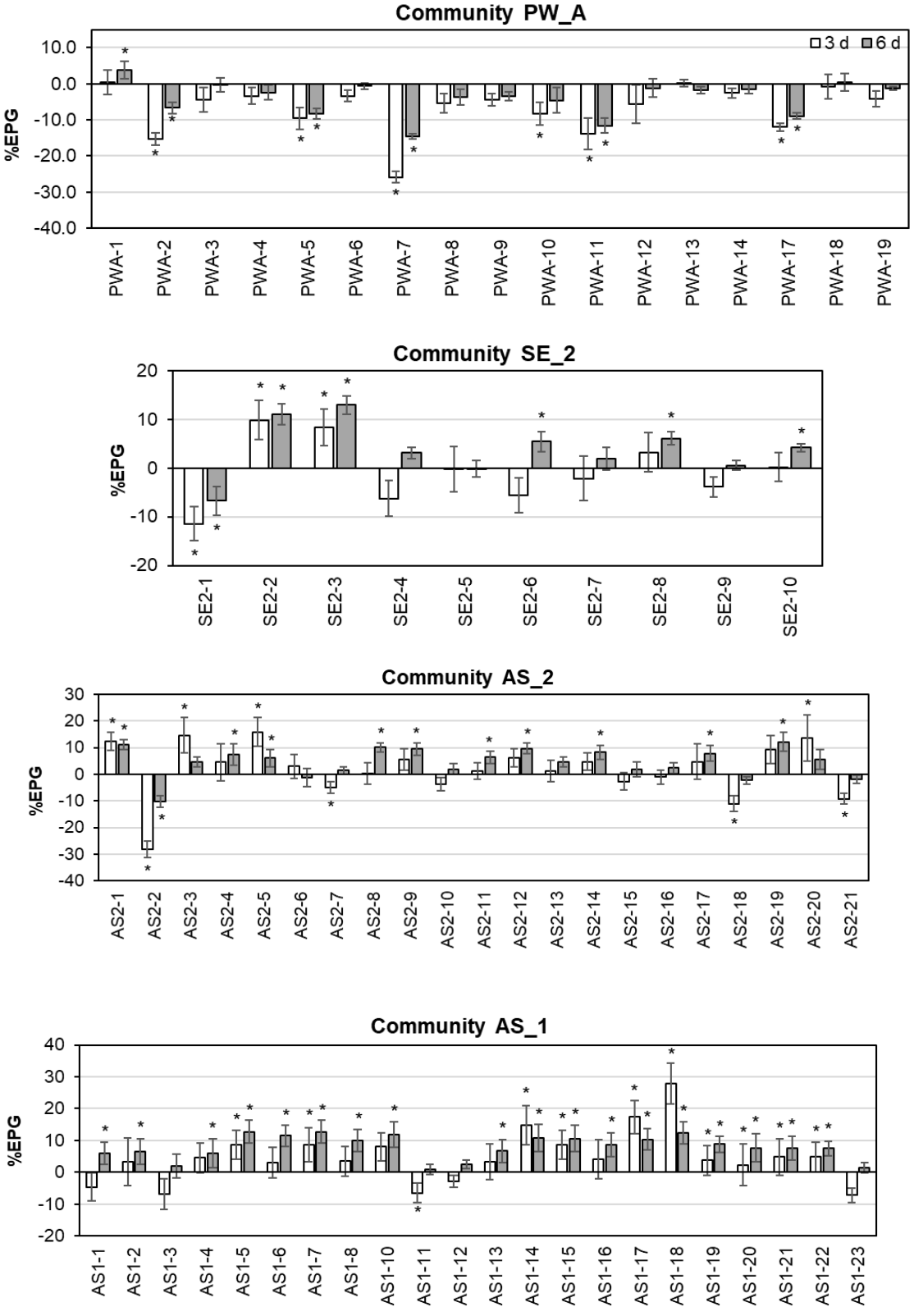


Fig. 3. 2 Effect on plant growth (EPG) of strains isolated from growth-promoting microbiomes after 3 and 6 days. Error bars represent standard deviations (n = 5). Asterisks indicate significant differences (one-way ANOVA, Tukey post hoc test; $p < 0.05$).

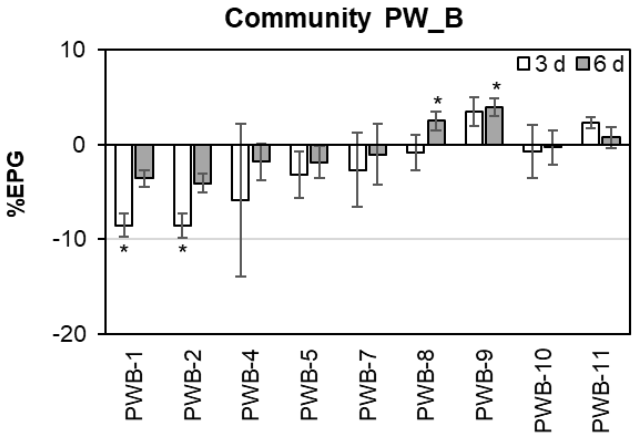


Fig. 3. 3 Effect on plant growth (EPG) of strains isolated from neutral microbiomes after 3 and 6 days. Error bars represent standard deviations (n = 5). Asterisks indicate significant differences (one-way ANOVA, Tukey post hoc test; $p < 0.05$).

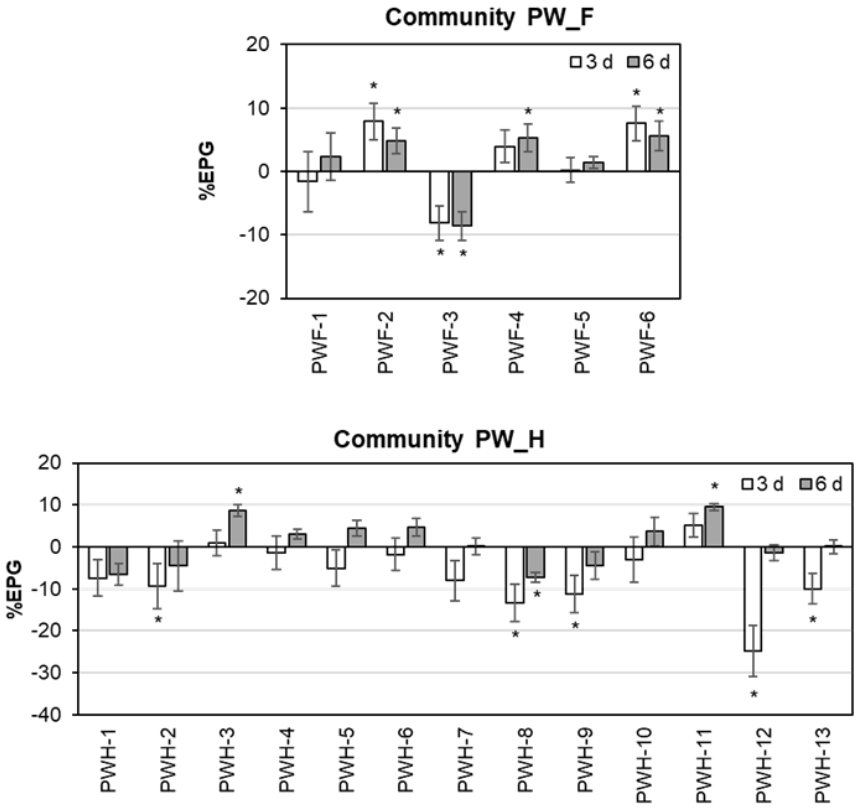


Fig. 3. 4 Effect on plant growth (EPG) of strains isolated from growth-inhibiting microbiomes after 3 and 6 days. Error bars represent standard deviations (n = 5). Asterisks indicate significant differences (one-way ANOVA, Tukey post hoc test; $p < 0.05$).

Table 3. 2 List of DABs isolated from *W. globosa* microbiomes function as proposed PGPB and plant PGIB. P, phosphate solubilization; Sid, siderophore production; HCN, hydrogen cyanide production; IRCs, indole-related compounds; %Sim, %similarity of 16S rRNA gene to closest taxon.

PGPB

Strain	3 d EPG (%)	6 d EPG (%)	Closest taxon	Acession	%Sim	Core ASV	Closest ASV	P	Sid	HCN	IRCs	IRCs type	IRCs Conc. (μg/mL)
SE2-2	9.8±4.0	11.1±2.2	*Agrobacterium pusense*	LC896520	100	○	○	+	-	-	+	Brown	5.4
SE2-3	8.3±3.8	12.9±1.9	*Brucella cytisi*	LC896521	99.77	○	○	-	+	-	+	orange	0.4
AS1-5	8.7±4.6	12.7±3.6	*Bosea spartocytisi*	LC896435	99.85	●	ASV_0085	-	-	-	-	No change	0
AS1-7	8.6±5.4	12.8±3.5	*Bosea beijingensis*	LC896437	99.56	●	ASV_0085	-	-	-	+	Pink	31.8
AS1-14	14.8±6.2	10.7±4.3	*Bosea beijingensis*	LC896443	99.77	●	ASV_0085	-	+	-	+	Pink	82
AS1-15	8.7±4.4	10.6±4.1	*Herbaspirillum aquaticum*	LC896444	99.86	●	ASV_0120	+	-	-	+	Pink	34.2
AS1-17	17.4±5.1	10.3±3.4	*Bosea beijingensis*	LC896446	99.62	●	ASV_0085	-	+	-	+	orange	3.1
AS1-18	27.8±6.4	12.3±3.5	*Bosea spartocytisi*	LC896447	99.85	●	ASV_0085	+	+	+	+	Brown	1.7
AS2-1	12.4±3.5	11.2±1.9	*Shinella curvata*	LC896453	99.63	●	ASV_0183	-	-	-	+	Brown	6.9
AS2-5	15.8±5.4	6.0±3.2	*Methylorubrum populi*	LC896457	99.93	○	○	-	-	-	+	Pink	8.4

PGIB

Strain	3 d EPG (%)	6 d EPG (%)	Closest taxon	Acession	%Sim	core ASV	Closest ASV	P	Sid	HCN	IRCs	IRCs type	IRCs Conc. (μg/mL)
PWA-2	-15.2±1.7	-6.7±1.5	*Novosphingobium huizhouense*	LC896475	99.48	●	ASV_0342	+	-	-	+	pink	70.7
PWA-5	-9.5±3.0	-8.3±1.5	*Methylophilus quaylei*	LC896478	99.78	●	ASV_0024	-	+	-	-	No change	0
PWA-7	-25.9±1.6	-14.6±0.8	*Novosphingobium huizhouense*	LC896480	99.48	●	ASV_0342	+	+	-	+	orange	1.2
PWA-11	-13.9±4.4	-11.6±2.1	*Methylophilus quaylei*	LC896484	99.78	●	ASV_0024	-	-	-	-	No change	0
PWA-17	-11.9±1.1	-8.9±0.8	*Phenylobacterium haematophilum*	LC896488	99.93	○	○	-	-	-	+	pink	64.5
SE2-1	-11.5±3.5	-6.8±2.9	*Brevundimonas vesicularis*	LC896519	99.52	○	○	-	-	+	+	Pink	36.9
AS2-2	-28.1±3.0	-10.2±2.2	*Burkholderia sola*	LC896454	100	○	○	+	+	-	-	No change	0
PWF-3	-8.1±2.7	-8.6±2.3	*Ralstonia mojiangensis*	LC896502	100	○	○	-	-	-	+	orange	1.1
PWH-8	-13.3±4.4	-7.3±1.2	*Methylobacterium goesingense*	LC896513	99.7	○	○	-	-	-	+	pink	50.6

Core PGPB strains, such as *B. beijingensis* (AS1-7, AS1-14, and AS1-17), *B. spartocytisi* (AS1-5 and AS1-18), *S. curvata* AS2-1, and *H. aquaticum* AS1-15, isolated from microbiomes AS_1 and AS_2, likely play critical roles in promoting *Wolffia* growth and maintaining microbiome stability. They likely serve as key determinants of *Wolffia* growth and represent core members, thereby confirming "positive key players." In general, core microorganisms interact closely with the host because they are more effectively adapted to the unique ecological challenges or contexts faced by the host species, thereby serving as a selective force. Previous studies on terrestrial plants have demonstrated that growth-promoting core strains outperform non-core strains in terms of reproducibility, transmission of beneficial functions, and competitive advantage over indigenous bacteria (Feng et al., 2024; Toju et al., 2018; Zhou et al., 2024). Previously reported PGPB inoculants have faced challenges in establishing strong associations with duckweed because of their microbiome member-selection behavior (Ishizawa et al., 2020a; Khairina et al., 2021). Therefore, the core PGPB strains identified in this study were assumed to establish a stronger relationship with *Wolffia* and promote better growth.

To the best of our knowledge, this study is the first to report *Bosea* spp. as PGPB in duckweed, specifically in *Wolffia*. Importantly, no evidence of human pathogenicity has been documented to date (Gu et al., 2024; Pulido-Suárez et al., 2022). Consequently, PGPB in *Bosea* spp. may be utilized in natural environments; however, additional research, encompassing genome-based functional profiling, pathogenicity assays, and assessment of their roles in plant interactions, is required. In addition, *S. curvata* AS2-1 serves as an effective PGPB in *Wolffia*. *Shinella curvata* was previously identified as a PGPB in *S. polyrhiza,* a rooted duckweed (Boonmak et al., 2023), indicating its potential effectiveness as a PGPB in both rooted and rootless duckweed species.

Evaluation at the community-level

In a summary of the effects of isolated DABs based on their original microbiomes (Table 3.1), strains isolated from growth-promoting microbiomes (PW_A, SE_2, AS_1, and AS_2) exhibited effects ranging from strongly inhibitory to strongly promotive on *Wolffia* growth, with EPG values varying between –14.6% and 12.9% after 6 days (Fig. 3.2). Most strains from SE_2, AS_1, and AS_2 exhibited enhanced host growth. Strains from AS_1 and AS_2 demonstrated more pronounced promotive effects after 6 days; however, microbiome AS_2 also contained strains with strong inhibitory effects, such as AS2-2, which consistently

suppressed *Wolffia* growth over 6 days (Fig. 3.2). Therefore, these microbial sources with growth-promoting effects are likely abundant in beneficial microbes and may function as effective inocula for establishing a superior plant microbiome or exploring PGPB, despite also containing PGIB. Surprisingly, in PW_A, most isolates demonstrated inhibitory effects, and no DAB with growth-promoting effects was identified (Fig. 3.2, Fig. 3.3), contrasting sharply with the community-level effect observed in PW_A (Fig. 2.1).

In microbiome PW_B, which exhibited a neutral effect, most of the nine isolated strains did not significantly influence *Wolffia* growth (Fig. 3.3, Fig. 3.5). Although the effects of PWB-8 and PWB-9 were statistically significant ($p < 0.05$), they remained within the neutral effect threshold (–5 to +5%). These findings infer that this microbiome ultimately exerts a neutral influence on host growth owing to the negligible effects of individual community constituents.

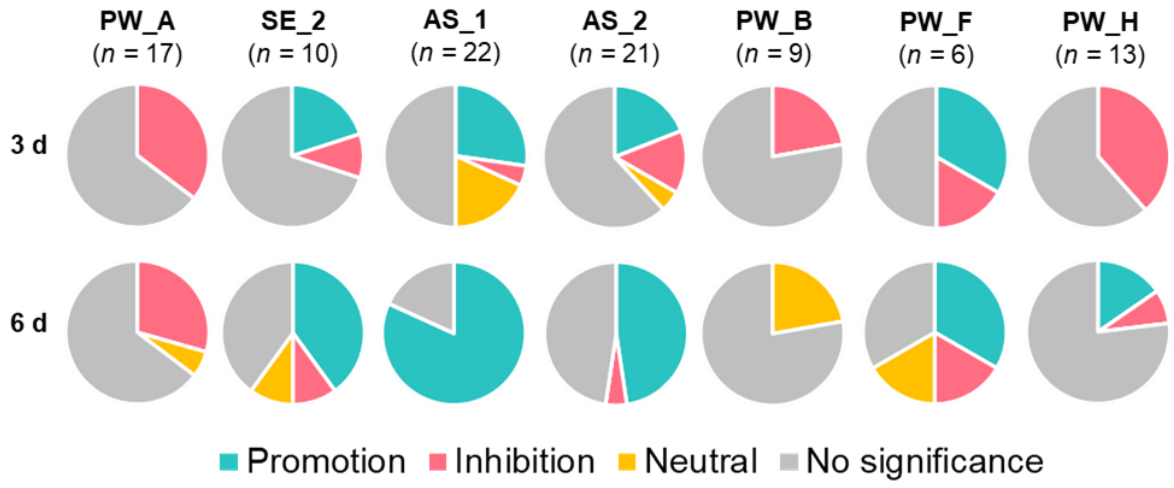


Fig. 3. 5 Proportion of isolated DABs within each original microbiome showing significant promotive (higher than +5%), neutral (–5% to +5%, but significant effect), and inhibitory effects (lower than –5%), or no significant effect compared with the control after 3 and 6 days.

Among the growth-inhibitory microbiomes (PW_F and PW_H), strains from PW_H generally suppressed *W. globosa* growth after 3 days; however, their inhibitory effects were mitigated after 6 days (Fig. 3.4, Fig. 3.5). Notably, PWH-12 initially showed a strong inhibitory effect (–24.8 ± 6.2 %), which shifted to neutral (–1.3 ± 1.9 %) after 6 days (Fig. 3.4). In contrast, among the six strains isolated from PW_F, most strains tended to slightly promote host growth,

whereas only PWF-3 consistently exhibited inhibitory effects (Fig. 3.4). This may be attributed to the small number of isolates from PW_F.

Overall, the findings of this study demonstrate that the effects of individual DABs on *W. globosa* growth are diverse and dynamic, differing not only among microbiomes but also among strains within the same community. The disproportionate presence of beneficial and harmful bacteria in growth-promoting and growth-inhibiting microbiomes, as exemplified by communities AS_1, AS_2, and PW_H, indicates that the net community effect depends on the balance between these groups rather than on a limited number of strong-effect strains, although the potential influence of uncultured DABs with extremely strong growth-promoting/inhibiting remains a possibility. Furthermore, microbiomes with higher diversity (e.g., diversity degree; Table 3.1) are more likely to contain beneficial microbes, thereby positively influencing host phenotype and productivity (Barelli et al., 2020; Coyte et al., 2015; Hu et al., 2016; Laurich et al., 2024).

Evaluation based on taxa

In addition to evaluating the effects of the isolated DABs on *Wolffia* growth at both the individual strain and community levels as the sum of all isolated strains, the effects of the isolated DABs were analyzed according to taxonomic group to better understand the relationship between bacterial taxa and *Wolffia* growth.

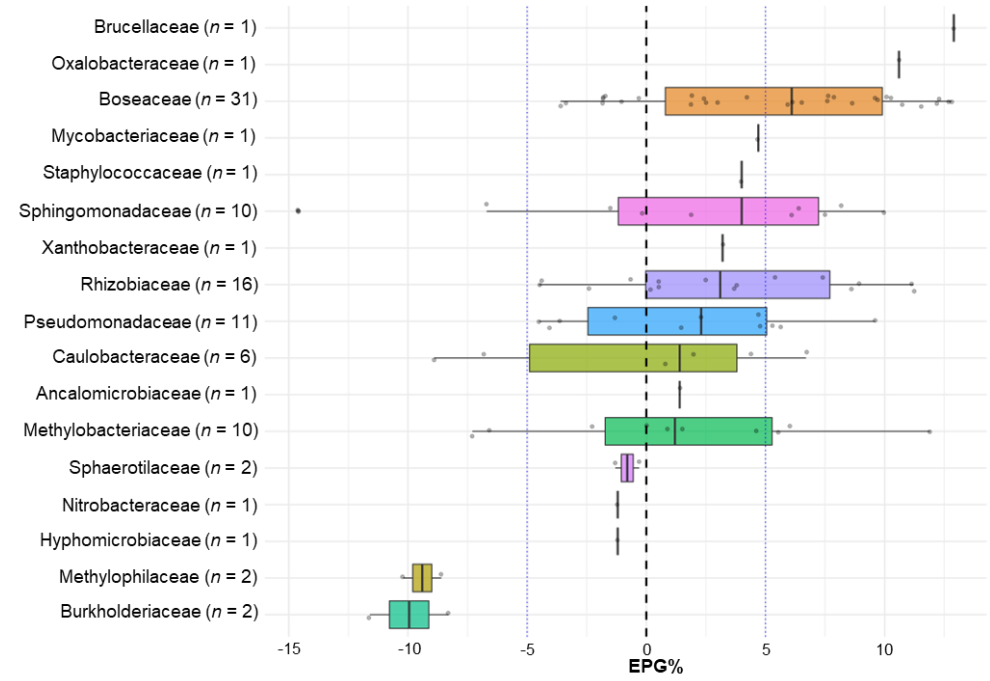
Figure 3.6 illustrates the range of EPG values for 98 DAB strains classified at the family and genus levels. Of the 17 families, 12 exhibited positive EPG values, whereas 5 showed negative EPG values. *Brucellaceae, Oxalobacteraceae*, and *Boseaceae* had mean EPGs higher than +5%. Conversely, *Burkholderiaceae* and *Methylophilaceae* uniformly inhibited host growth, with mean EPGs lower than –5%. Notably, isolates of *Rhizobiaceae* and *Pseudomonadaceae* predominantly exhibited positive effects on *Wolffia* growth, with no inhibitory effects observed at EPG < –5% (Fig. 3.6a; Fig. 3.7a). *Sphingomonadaceae, Caulobacteraceae*, and *Methylobacteriaceae* included isolates with both positive and negative EPGs. Notably, promotive effects were predominant in *Sphingomonadaceae* and *Methylobacteriaceae*, whereas inhibitory effects were predominant in *Caulobacteraceae* (Fig. 3.6a; Fig. 3.7a). These findings are consistent with those in Chapter 2 derived from 16S rRNA amplicon sequencing, indicating that *Wolffia* growth is positively correlated with *Rhizobiaceae*, *Boseaceae*, and *Sphingomonadaceae* and negatively correlated with *Burkholderiaceae* and *Caulobacteraceae*. Interestingly, the opposite trend was observed for *Pseudomonadaceae*

when comparing the previous culture-independent study with the current culture-based study. This discrepancy may indicate microbe-microbe interactions or ecological context dependence (Laurich et al., 2024), although the precise interactions remain unclear.

Among the 30 genera, *Brucella*, *Herbaspirillum*, *Shinella*, *Agrobacterium*, *Sphingopyxis*, *Bosea*, and *Metapseudomonas* were particularly effective in promoting *Wolffia* growth (Fig. 3.6b; Fig. 3.7b). Conversely, all isolates belonging to *Burkholderia*, *Methylophilus*, *Ralstonia*, and *Brevundimonas* and more than half of the *Novosphingobium* isolates largely inhibited *Wolffia* growth. Notably, members of *Novosphingobium* and *Brevundimonas* have been identified as PGPB in rooted duckweed, such as *Novosphingobium* sp. W5-13 (Boonmak et al., 2023), *Novosphingobium* sp. KLaS07, and *Brevundimonas* sp. KLaSR13 (Thanh Phạm et al., 2025). However, these genera were identified as PGIB in *Wolffia*, indicating that their functional roles may be affected by host morphology or host-secreted metabolites. Meanwhile, *Methylophilus* was reported as a PGIB in duckweed for the first time.

Genus comparison demonstrated superior predictive power to family comparison: genera with inhibitory effects demonstrated 100% phenotypic consistency across all strains (e.g., *Burkholderia* and *Ralstonia* within Burkholderiaceae and *Methylophilus* within Methylophilaceae) (Fig. 3.6b; Fig. 3.7b), whereas some families with promotive effects (*Sphingomonadaceae*, *Methylobacteriaceae*, and *Caulobacteraceae*) retained 20–40% inhibitory strains (Fig. 3.7a). Meanwhile, in genera with a high proportion of promotive effects (e.g., *Bosea*, n = 31; *Rhizobium*, n = 10; *Metapseudomonas*, n = 8), substantial phenotypic heterogeneity persisted (Fig. 3.7b). This indicates that strain-level characteristics, rather than taxonomic rank, ultimately determine plant growth effects. This asymmetry may indicate that inhibitory phenotypes are conserved family-level traits, whereas promotive phenotypes are strain-specialized traits. This establishes a predictive hierarchy: family serves as a broad classification, genus provides an intermediate prediction, and strain offers a definitive classification.

a) (ANOVA, p <0.05, n = 17 families, 98 strains)



b) By genus (ANOVA, p <0.05, n = 30 genera, 98 strains)

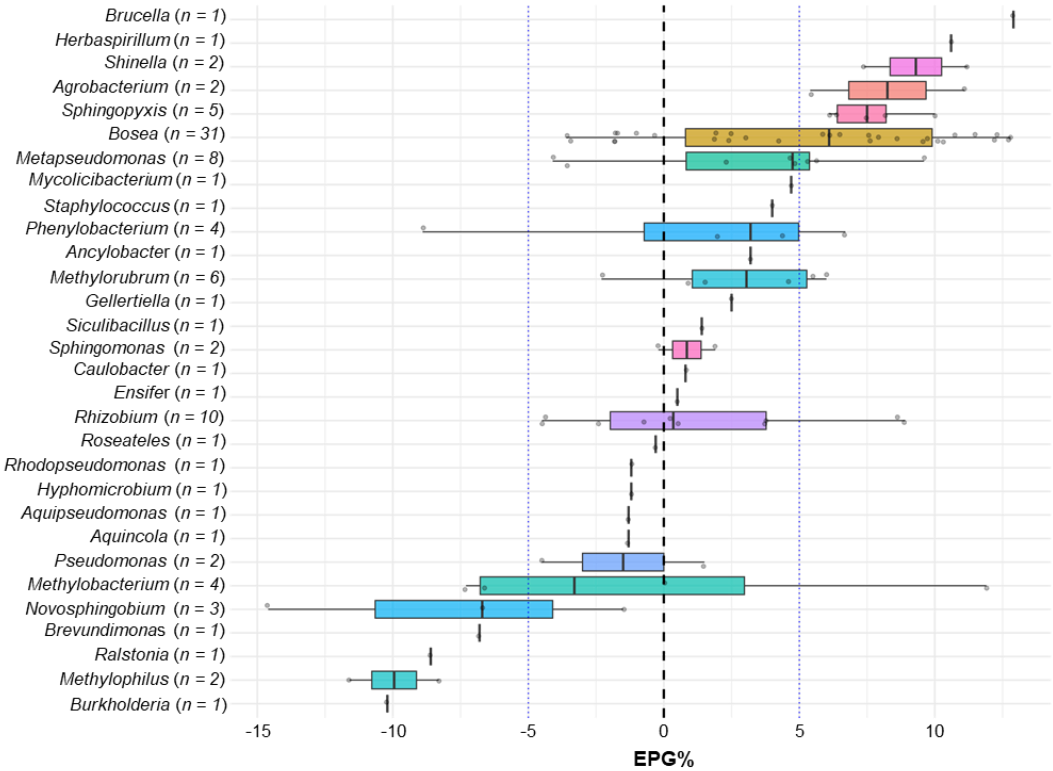
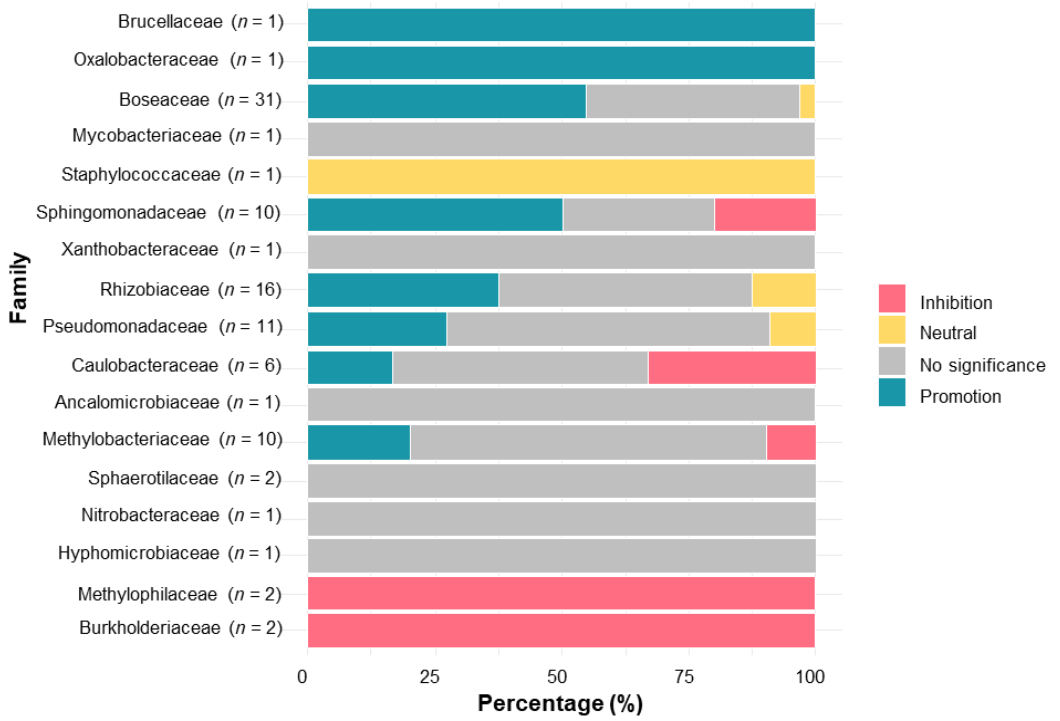


Fig. 3. 6 Effect on plant growth of 98 DABs after 6-day cultivation term, categorized by taxa: a) by bacterial family, b) by genus. Error bars represent standard deviations within each genus (n indicated in the figure). Black dashed line indicates growth relative to that of the control. Blue dashed line marks the EPG threshold for a neutral effect.

a) Family



b) Genus

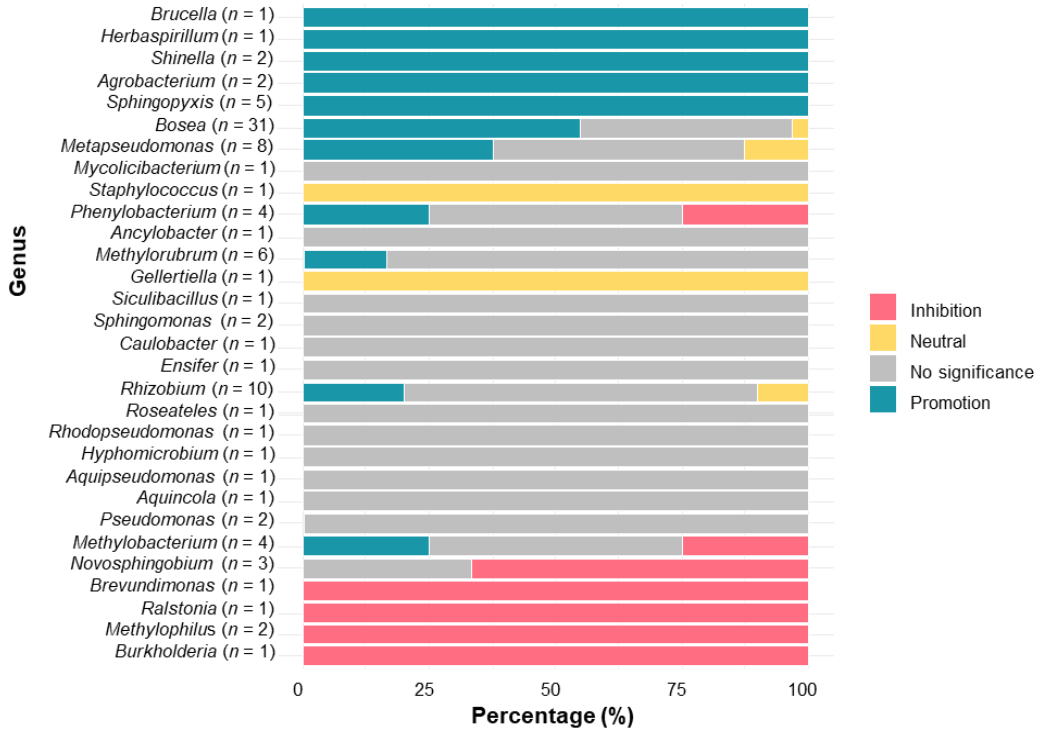


Fig. 3. 7 Distribution of the effects of 98 DABs on *W. globosa* growth after 6 days, categorized by (a) family and (b) genus.

3.3.3 Physiological characterization of DABs affecting Wolffia growth

The abilities of all isolates to produce siderophores, IRCs, and HCN, and solubilize phosphate were assessed as representative physiological characteristics associated with host plant growth. Among the 98 strains, 88 strains (89.8%) were positive for at least one trait, with only one strain positive for all four traits (Table 3.3). Figure 3. 8 shows the proportions of positive strains for each trait. Specifically, 24 strains (24.4%) demonstrated phosphate solubilization activity, 26 strains (26.5%) produced siderophores (Fig. 3.9), and 6 strains (6.1%) were positive for HCN production Notably, 85 strains (86.7%) were capable of synthesizing IRCs at concentrations ranging from 0.1 to 175.4 µg/mL. Based on colorimetric detection and wavelength scanning, the IRCs were classified into three types: pink (48 strains), orange (26 strains), and brown (11 strains) (Fig. 3.10). Although precise identification requires liquid chromatography-mass spectrometry analysis, the pink type is suspected to be indole-3-acetic acid (IAA), indole-acetamide, or indole-3-pyruvic acid; the orange type is likely to be indole-3-butyric acid (IBA); and the brown type is thought to be indole (Gilbert et al., 2022; Gilbert et al., 2018).

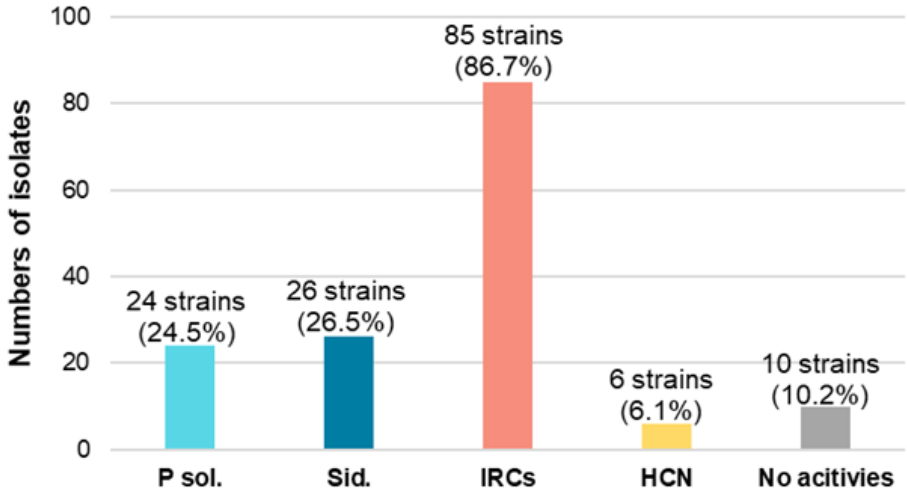


Fig. 3. 8 Numbers of DABs that showed abilities in phosphate solubilization, siderophore production, hydrogen cyanide production, and IRCs production, and no ability.

Table 3. 3 Physiological characteristics of 98 DABs isolated from *Wolffia* microbiomes.

Strains	P sol.	Sid.	HCN	IRCs conc. (ug/mL)	Type of IRCs.
Growth-promoting microbiomes					
Community PW_A					
PWA-1	+	-	-	13.6	Orange
PWA-2	-	-	-	50.6	Pink
PWA-3	-	-	-	64	Pink
PWA-4	-	-	-	62	Pink
PWA-5	-	-	-	64.5	Pink
PWA-6	+	-	-	59.8	Pink
PWA-7	+	-	-	70.7	Pink
PWA-8	-	-	-	-	NC
PWA-9	-	-	-	77.2	Pink
PWA-10	+	-	-	51.1	Pink
PWA-11	-	+	-	-	NC
PWA-12	-	-	-	9.9	Orange
PWA-13	-	-	-	7.6	Orange
PWA-14	-	-	-	63.4	Pink
PWA-17	-	-	-	-	NC
PWA-18	+	-	-	14.3	Orange
PWA-19	+	-	-	5.8	Orange
Community SE_2					
SE2-1	-	-	+	36.9	Pink
SE2-2	+	-	-	34.2	Pink
SE2-3	+	-	-	5.4	Brown
SE2-4	-	-	+	14.2	Pink
SE2-5	-	-	-	37.1	Pink
SE2-6	+	-	-	48.2	Pink
SE2-7	-	-	-	33.8	Pink
SE2-8	-	-	-	22.7	Pink
SE2-9	-	-	-	6.3	Orange
SE2-10	-	-	-	-	NC
Community AS_1					
AS1-1	-	-	-	8.6	Brown
AS1-2	-	-	-	17.3	Pink
AS1-3	-	-	-	47.4	Orange
AS1-4	-	-	-	4.9	Orange
AS1-5	-	-	-	-	NC
AS1-6	-	-	-	1.1	Brown
AS1-7	-	+	-	0.4	Orange
AS1-8	+	+	-	25.1	Pink
AS1-10	-	-	+	45.4	Pink
AS1-11	+	-	-	10	Orange
AS1-12	-	+	-	30	Pink
AS1-13	-	-	-	59.2	Pink
AS1-14	-	+	-	3.1	Orange
AS1-15	+	+	+	1.7	Brown
AS1-16	+	+	-	18.4	Pink
AS1-17	-	-	-	6.9	Brown
AS1-18	-	-	-	31.8	Pink
AS1-19	+	+	-	2.2	Orange
AS1-20	+	+	-	29.3	Pink
AS1-21	+	-	+	47.1	Pink
AS1-22	-	-	-	0.2	Orange
AS1-23	-	-	-	-	NC
Community AS_2					
AS2-1	-	+	-	82	Pink
AS2-2	+	+	-	1.2	Orange
AS2-3	-	+	-	16.4	Pink
AS2-4	-	-	-	111.1	Pink
AS2-5	-	-	-	8.4	Pink
AS2-6	-	-	-	18.9	Pink
AS2-7	+	-	-	2.2	Orange
AS2-8	-	-	-	26.5	Pink
AS2-9	-	-	-	21.6	Pink
AS2-10	-	-	-	6	Brown
AS2-11	+	-	-	25	Orange
AS2-12	-	-	-	35.8	Orange
AS2-13	-	+	-	45.6	Orange
AS2-14	-	-	-	33.3	Brown
AS2-15	-	-	+	47.3	Brown
AS2-16	-	-	-	0.6	Orange
AS2-17	-	-	-	31.4	Brown
AS2-18	-	-	-	45.8	Brown
AS2-19	-	-	-	40.5	Orange
AS2-20	-	-	-	38.4	Brown
Neutral microbiome					
Community PW_B					
PWB-1	-	+	-	71.7	Pink
PWB-2	-	+	-	32.5	Pink
PWB-4	-	-	-	-	NC
PWB-5	-	-	-	-	NC
PWB-7	-	-	-	24.7	Pink
PWB-8	-	-	-	0.1	Orange
PWB-10	-	-	-	-	NC
PWB-11	-	-	-	2.3	Orange
Growth-inhibiting microbiomes					
Community PW_F					
PWF-1	-	+	-	71.7	Pink
PWF-2	-	+	-	67.7	Pink
PWF-3	+	+	-	-	NC
PWF-4	-	+	-	53	Pink

Strains	P sol.	Sid.	HCN	IRCs conc. (ug/mL)	Type of IRCs.	Strains	P sol.	Sid.	HCN	IRCs conc. (ug/mL)	Type of IRCs.
PWF-5	+	-	-	-	NC	PWH-6	-	+	-	67.3	Pink
PWF-6	-	+	-	91.8	Pink	PWH-7	-	-	-	81.6	Pink
Community PW_H						PWH-8	-	-	-	1.1	Orange
PWH-1	-	-	-	1.8	Orange	PWH-9	-	-	-	67.3	Pink
PWH-2	+	+	-	17.8	Pink	PWH-10	-	-	-	-	NC
PWH-3	-	+	-	70	Pink	PWH-11	-	-	-	175.4	Pink
PWH-4	-	-	-	-	NC	PWH-12	-	+	-	7.7	Pink
PWH-5	+	+	-	21.2	Pink	PWH-13	-	+	-	1.2	Orange

*NC, No color change

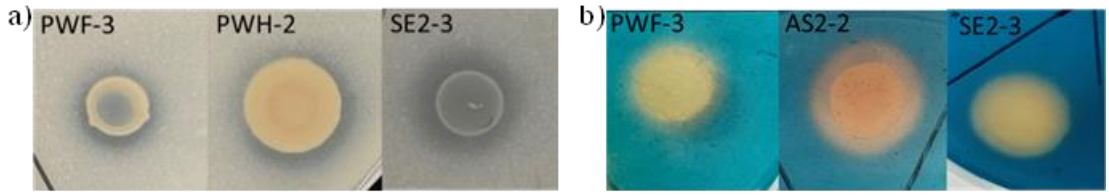


Fig. 3. 9 Positive in plant growth-promoting traits of strains, a) phosphate solubilization test in Pikovskaya's medium, b) siderophore production test in Chrome Azural S medium.



Fig. 3. 10 Color change of Salkowski's reagent after reaction with supernatants of DAB culture.

Statistical analysis with multiple-way ANOVA revealed that of the four traits tested, only IRC production was significantly correlated with *Wolffia* growth promotion ($p < 0.05$) (Table 3.4). Interestingly, additional analyses revealed that among the three types of IRCs, the orange type was negatively correlated with growth promotion ($p < 0.05$), whereas the pink and brown types showed no statistical significance ($p > 0.05$).

Table 3. 4 Correlation of four physiological traits of isolated strains with their effects on *W. globosa* growth, analyzed using multiple-way analysis of variance (ANOVA).

Factor	Mean square	F value	Coefficients	Significance
Phosphate solubilization	2.5	0.067	-0.51	0.796
Siderophore production	6.16	0.165	0.65	0.685
HCN production	18.27	0.49	1.82	0.486
Indole-related compounds (IRCs)	174.55	4.683	3.78	0.033
Pink type of IRCs	30.66	0.849	-4.01	0.359
Orange type of IRCs	183.78	5.087	-4.04	0.027
Brown type of IRCs	0.16	0.005	0.27	0.946

IRCs are involved in plant phytohormones that influence terrestrial plant growth. Recent studies have reported that DABs, including PGPB, associated with rooted duckweed produce IRCs, predominantly IAA (Boonmak et al., 2023; Khairina et al., 2021; Makino et al., 2022; Yamakawa et al., 2018). However, their role in duckweed growth remains contentious: external application of IAA [0–50 μM (0–8.76 μg/mL)] did not promote growth in *L. minor* (Utami et al., 2018), and no correlation was found between *L. minor* growth promotion and DAB-produced IAA (Ishizawa et al., 2017). In this study, a significant positive correlation between IRC production and *Wolffia* growth promotion was identified for the isolated DABs, highlighting the importance of IRCs for *Wolffia* growth. In contrast, the orange-type IRC, likely IBA, was negatively correlated with growth promotion in aquatic plants for the first time. IBA serves as a precursor to IAA and promotes lateral root formation (Frick and Strader, 2017). Research indicates that the IBA-producing bacterium *Azospirillum brasilense*, which yields purified IBA, promotes lateral root formation while simultaneously inhibiting root elongation in maize. This suggests that IBA induces tissue-specific responses (Martínez-Morales et al., 2003). Given the leaf-like structure (fronds) of *Wolffia*, the negative effects of IBA may reflect tissue-specific sensitivity. Among the DABs isolated in this study, two PGIB strains, *Burkholderia sola* AS2-2 and *Methylobacterium goesingense* PWH-8, and two PGPB strains, *B. beijingensis* AS1-7 and AS1-14, showed IBA-producing abilities. Therefore, the mechanism by which IBA influences *Wolffia* growth requires further investigation.

Although the other IRC types, pink (likely IAA) and brown (indole) types, did not demonstrate significant correlations with *Wolffia* growth promotion, their varying trends may indicate distinct roles in *Wolffia* growth. IAA was produced by multiple strains and exhibited a negative coefficient, whereas indole was produced by 11 strains, including 3 out of 10 PGPB,

with none produced in PGIB, and displayed a positive coefficient. This indicates a specific relationship between indole production and the interactions of certain beneficial bacteria with *Wolffia*. This finding is consistent with that of Gilbert et al. (2018, 2022), demonstrating that indole-producing DABs isolated from *Wolffia* positively affect the host plant and effectively colonize leaf surfaces. Indole acts as a bacterial signaling molecule involved in communication, plant–microbe interactions, biofilm formation, and quorum sensing (Martino et al., 2003; Spaepen and Vanderleyden, 2011), potentially facilitating the colonization on the leaf-like structure of *Wolffia*. In contrast, the prevalence of IAA production and its negative trend suggest a more complex role, given that IAA is a common auxin that regulates plant growth and bacterial colonization (Spaepen et al., 2007). Further investigations are needed to clarify its functional effects on plant growth and plant-microbe relationships.

3.4 Summary

In this chapter, a total of 98 DABs were isolated from 7 *Wolffia* microbiomes their individual effects on *Wolffia* growth were characterized. The isolated DABs had diverse effects on *Wolffia* growth, ranging from strong inhibition, neutral, or strong promotion. Based on the experimental results, 10 and 9 strains were listed as the candidates of PGPB and PGIB, respectively. Of these 19 strains, 7 PGPBs, including *Bosea beijingensis* (3 strains), *Bosea spartocytisi* (2 strains), *Shinella curvata* (1 strain), and *Herbaspirillum aquaticum* (1 strain), matched core ASVs that had been observed in respective microbiomes and were identified as "positive key players," significantly enhancing *Wolffia* growth. Meanwhile, 4 PGIBs, including *Methylophilus quaylei* (2 strains) and *Novospingobium huizhouense* (2 strains), were identified as "negative key players," strongly suppressing *Wolffia* growth. Further evaluation of the growth effects of isolated DABs at the community level indicates that the balance between beneficial and detrimental members within the microbiome determines the net community effect on *Wolffia* growth. It should also be noted that inhibiting phenotypes of DABs were conserved at the genus level (e.g., *Methylophilus* and *Burkholderia*), whereas promotive outcomes were heterogeneous and determined at the strain level (e.g., *Bosea* and *Shinella*). IRC production emerged as a key physiological determinant correlated with *Wolffia* growth. The overall findings obtained in this chapter are summarized in Fig. 3.11.

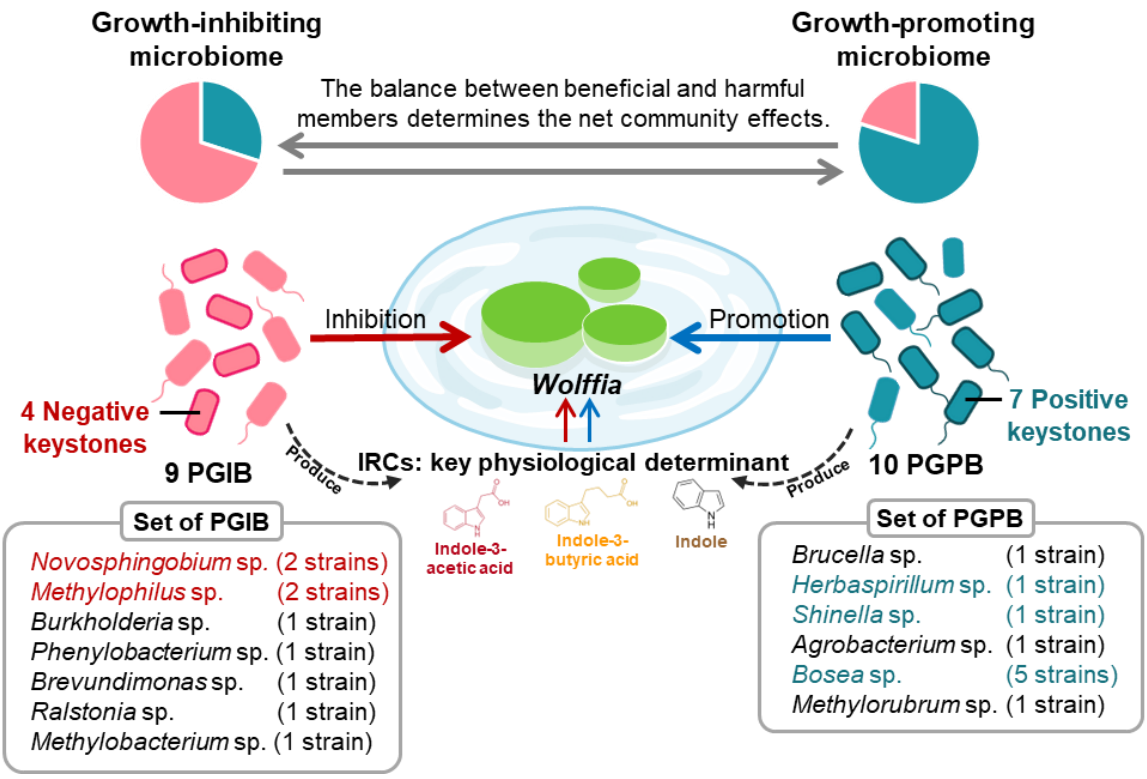


Fig. 3. 11 Overall findings from the characterization of DABs derived from *Wolffia* microbiomes.

Chapter 4 Summary and Conclusions

Duckweed has garnered attention as a promising bioresource due to its rapid growth, global distribution, and nutritionally valuable biomass composition. Previous studies have demonstrated that the growth and physiological state of duckweeds are significantly influenced by interactions with their symbiotic microbiome and specific DABs within it. Therefore, efforts have been made to characterize the phylogenetic structure and functional traits of the duckweed microbiome and elucidate their physiological and ecological functions of DABs. However, the majority of prior studies on the microbiomes have focused on rooted duckweeds (e.g., *Lemna* and *Spirodela*). Research on the microbiomes of *Wolffia*, which holds exceptionally great value as a bioresource and for which the establishment of efficient cultivation technologies is desired, is quite scarce. Given the unique morphology of *Wolffia*, the structure and function of its microbiome likely differ from those of rooted duckweeds, as suggested in limited preliminary reports. Consequently, a systematic characterization of the *Wolffia* microbiome to give insights into the enhanced *Wolffia* production holds significant importance.

Therefore, the overall objective of this study was to clarify the structure and function of the *Wolffia* microbiome and to identify DABs acting as PGPB and PGIB within *Wolffia* microbiome and their key traits influencing *Wolffia* growth. To these aims, this study conducted (i) characterization of structural and functional properties of bacterial communities which were assembled on *W. globosa* surface from various microbial sources and their effects on host growth, (ii) evaluation of the individual effects of culturable DABs within *Wolffia* microbiomes on *Wolffia* growth, and (iii) characterization of their physiological traits that may be associated with host plant growth promotion or inhibition.

In Chapter 1, duckweeds were introduced as the smallest and fastest-growing flowering plants, and *Wolffia* was highlighted as an exceptionally promising bioresource contributing to the bio-circular economy owing to its rapid biomass production and valuable nutritional profiles. Current strategies to enhance duckweed biomass production include advanced cultivation systems and microbiome-based approaches. Duckweeds form strong associations with the microbiome, comprising diverse DABs that can promote and inhibit duckweed growth. Earlier studies have demonstrated clear success in improving duckweed biomass by the use of beneficial environmental microbial communities and specific PGPB. However, most studies have focused on rooted duckweeds, particularly *Lemna* and *Spirodela*,

and current knowledge on the microbiome and DABs for *Wolffia* is very limited. Based on these gaps, the objectives of this thesis were set as described above.

In Chapter 2, the structural and functional properties of the *W. globosa* microbiome and its effects on host growth were investigated via co-cultivation with bacterial communities derived from ten freshwater pond samples, four municipal secondary effluent samples, and four activated sludge samples. The bacterial communities showed diverse effects on *Wolffia* growth, from inhibitory to strongly promotive, while municipal wastewater-derived communities generally exhibited stronger promotive effects than pond-derived communities. 16S rRNA amplicon sequencing revealed that *Wolffia* selectively assembled specific bacterial populations from each source, resulting in lower diversity and distinct community structures compared with the original microbial sources. Across all categories, *Wolffia* microbiomes were dominated by Proteobacteria and shared a set of core families: *Beijerinckiaceae, Caulobacteraceae, Comamonadaceae, Methylophilaceae, Rhizobiaceae*, and *Sphingomonadaceae*. Functional prediction indicated common enrichment of traits related to colonization and plant interaction, such as chemotaxis, flagella, biofilm formation, quorum sensing, secretion systems, and transporters. In addition, *Bdellovibrionaceae, Beijerinckiaceae*, and *Sphingomonadaceae* were identified as potential "keystone taxa," shaping community structure and directly or indirectly exerting a significant influence on *Wolffia* growth. Collectively, the findings in this chapter unveiled the robust core taxa and functional profiles of the *Wolffia* microbiome across diverse microbial sources, with certain taxa differing from those in rooted duckweed.

In Chapter 3, nearly 100 DABs were isolated from seven *Wolffia* microbiomes established in Chapter 2, and their taxonomies and functional traits associated with host plant growth were characterized. The isolated DABs were taxonomically diverse, belonging mainly to Pseudomonadota, and more than half matched core ASVs identified in *Wolffia* microbiomes, confirming that core taxa were successfully captured. Through co-cultivation assays employing individual DABs, ten strains were identified as PGPB candidates and nine as PGIB candidates, of which seven PGPB and four PGIB corresponded to core ASVs, respectively, suggesting that a small subset of core strains act as positive or negative key players in the *Wolffia* holobiont. At the community level, the growth-promoting microbiomes were enriched in growth-promoting strains, whereas the growth-inhibiting microbiomes contained more strains with neutral or negative effects on host plant growth. Taxonomic evaluation demonstrated that prominent promotive phenotypes occurred across seven genera; however,

their plant growth effects depend on the strain-level characteristics. On the other hand, inhibitory phenotypes were conserved at the genus level. IRC production by DABs emerged as a key physiological determinant correlated with *Wolffia* growth. The findings of this chapter provide a traceable set of positive and negative key players, significantly influencing *Wolffia* growth: *B. beijingensis*, *B. spartocytisi, S. curvata,* and *H. aquaticum* as positive key players, and *Me. quaylei* and *N. huizhouense* as negative key players.

In conclusion, this study demonstrated that *Wolffia* selectively assembles its microbiome members from surrounding environments, with microbiome composition varying by microbial sources, while a robust set of core taxa is conserved across all microbial sources. In turn, the microbiomes modulate *Wolffia* growth, from strong promotion to clear inhibition, which is determined by the balance between PGPB and PGIB within the microbiomes. This study also identified IRC production of DABs as a key physiological trait associated with *Wolffia* growth, and the candidates of positive and negative key players in *Wolffia* holobiont with a strong modulating effect on *Wolffia* growth.

From a broader perspective, the insights gained in this study advance understanding of *Wolffia*–microbiome interactions and offer practical clues on microbiome engineering strategies for developing efficient biomass production systems. Specifically, it is suggested that *Wolffia* cultivation can be enhanced by (i) using carefully selected environmental microbial sources, such as secondary effluent and activated sludge, (ii) enriching or introducing positive key players while suppressing negative key players, and (iii) considering indole-related signaling as a central axis in the design of compatible plant–microbe partnerships.

Despite the systematic characterization of the *Wolffia* microbiome and DABs in this study, further studies are required to design scalable, effective, and practically applicable technologies to enhance *Wolffia* biomass production. In addition, deeper investigations of *Wolffia*–microbiome interactions are still needed. The following future challenges are proposed:

(i) Although this study successfully investigated the effects of diverse bacterial communities and characterized the *Wolffia* microbiome, the effects of bacterial abundance in the original microbial sources and various physicochemical parameters on shaping the microbiome remain unclarified.

(ii) The relationships between the dynamics of DABs and *Wolffia* growth represent a critical question for subsequent research, which requires the application of quantitative assessments of DABs abundance and their gene expressions.

(iii) The detailed mechanisms for *Wolffia* growth promotion by the identified PGPB, especially positive key players, should be clarified, including precise identification of IRC types which requires liquid chromatography–mass spectrometry analysis.

(iv) The fundamental growth characteristics of PGPB identified in this study, including the effects of environmental parameters, were to be evaluated to determine their optimum applied conditions to maximize the growth-promoting effects.

(v) This study focused on the effects of microbiomes and DABs on *Wolffia* growth based on the frond area. However, given the significance of *Wolffia* as the bioresource, further investigations focusing on their effects on *Wolffia* biomass quality (e.g., protein and starch content, and other valuable nutrients) are necessary.

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Achievements

Publication related to this thesis

1. **Saimee, Y.**, Kuwai, K., Ishizawa, H., Inoue, D., Thamchaipenet, A. and Ike, M. 2025. Structural and functional properties of bacterial communities associated with rootless duckweed (*Wolffia globosa*) and their effect on the *Wolffia* growth. Environmental Microbiome, 20(1), 102. https://doi.org/10.1186/s40793-025-00759-6.

2. Ishizawa, H., **Saimee, Y.**, Sugiyama, T., Kojima, T., Inoue, D., Ike, M., Thamchaipenet, A. and Morikawa, M. 2025. Duckweed as an emerging model system for plant–microbiome interactions. Environmental Microbiology, 27(9), e70181. https://doi.org/10.1111/1462-2920.70181.

3. **Saimee, Y.**, Okada, R., Inoue, D., and Ike, M. 2025. Characterization of duckweed-associated bacteria from the *Wolffia* microbiome: strain-level effects on host growth and key determinants. Journal of Plant Growth Regulation (Under review).

4. **Saimee, Y.**, Ishizawa, H., Inoue, D., and Ike, M. 2025. Commentary: From water to frond – *Wolffia* shapes its microbiome to drive its growth. Duckweed Forum, 14(1), 2. http://www.ruduckweed.org/uploads/1/0/8/9/10896289/duckweed_forum_issue_52.pdf.

Other Publications

1. **Saimee, Y**. and Duangmal, K. 2021. *Streptomyces spirodelae* sp. nov., isolated from duckweed. International Journal of Systematic and Evolutionary Microbiology, 71(11), 005106. https://doi.org/10.1099/ijsem.0.005106.

2. **Saimee, Y.**, Butdee, W., Boonmak, C. and Duangmal, K. 2024. *Actinomycetospora lemnae* sp. nov., A novel actinobacterium isolated from *Lemna aequinoctialis* able to enhance duckweed growth. Current Microbiology, 81(3), 92. https://doi.org/10.1007/s00284-023-03595-4.

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International Conferences

1. **Saimee, Y.**, Kuwai, K., Okada, R., Inoue, D. and Ike, M. Impact of bacterial communities on the growth of rootless duckweed (*Wolffia globosa*) and their recruited bacterial community from surrounding environment. The 13th International Conference on Environmental Engineering, Science and Management (13th EETA), Khon Kaen, Thailand, May 2024. Oral presentation.

2. **Saimee, Y.**, Kuwai, K., Ishizawa, H., Inoue, D., and Ike, M. Taxonomical structure and functional properties of *Wolffia* microbiome and their effect on *Wolffia* growth. The 7th International Conference on Duckweed Research and Applications (7th ICDRA), Bangkok, Thailand. November 2024. Poster Presentation.

3. Boonchu, P., **Saimee, Y.**, Kammanee, S., Duangmal, K. Actinobacteria isolated from *Spirodela polyrhiza* and their plant growth-promoting activity to enhanced duckweed growth. The 7th International Conference on Duckweed Research and Applications (7th ICDRA), Bangkok, Thailand. November 2024. Poster Presentation.

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5. Kuwai, K., **Saimee, Y.**, Okada, R., Inoue, D. and Ike, M. Structural and functional properties of bacterial communities associated with rootless duckweed (*Wolffia globosa*) and their effect on the *Wolffia* growth. Water and Environment Technology Conference 2025 (WET2025), Niigata, Japan. July 2025. Poster presentation.

6. **Saimee, Y.**, Kuwai, K., Ishizawa, H., Inoue, D., Thamchaipenet, A. and Ike, M. Impact of environmental bacterial communities on the growth of rootless duckweed (*Wolffia*

globosa) and *Wolffia* microbiome structure. The 17th International Joint Workshop on Advanced Engineering Technology for Environment and Energy (The 17th AETEE). Shenyang, China. August 2025. Oral presentation.

Domestic Conferences

1. Kuwai, K., **Saimee, Y.**, Okada, R., Inoue, D. and Ike, M. Phylogenetic properties of *Wolffia globosa* microbiomes and their relation to biomass production. The 58th Annual Conference of Japan Society on Water Environment (JSWE), Fukuoka, Japan. March 2024. Poster presentation.

Other presentations

1. **Saimee, Y.**, Kuwai, K., Okada, R., Inoue, D. and Ike, M. 2024. Establishment of duckweed-microbial community system for valuable biomass production. Progress report 2024 for the SATREPS project under Be-HoBiD, Department of Microbiology, Kasetsart University, Bangkok, Thailand.
2. **Saimee, Y.**, Kuwai, K., Ishizawa, H., Inoue, D., and Ike, M. 2025. Establishment of *Wolffia*-microbial holobiont system for valuable biomass production. Progress report 2025 for the SATREPS project under Be-HoBiD, Kasetsart University, Bangkok, Thailand.

Awards

1. First-class honors degree, B.Sc. in Biology, Nakhon Pathom Rajabhat University, 2019
2. Award for Outstanding Academic Achievement at the Bachelor of Science Level, The Professor Dr. Tab Nilanidhi Foundation, 2019.
3. Popular award for poster presentation of The 7th International Conference on Duckweed Research and Applications (ICDRA), 2024.
4. Best oral presentation award of The 17th International Joint Workshop on Advanced Engineering Technology for Environment and Energy (AETEE), 2025.
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