

Article

Exploring the Cultivable Fraction of the Bacterial Microbiome from Tomato Plants for Growth-Promoting and Biocontrol Traits Toward Bioinput Development

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Abstract

Plant growth-promoting bacteria (PGPB) represent a sustainable alternative to synthetic inputs in horticultural systems; however, their bioprospecting is hindered by the absence of integrative, performance-oriented selection strategies. In this study, a comprehensive collection of 259 bacterial isolates associated with tomato plants was systematically screened to identify strains with biocontrol and plant growth-promoting potential. Isolates were characterized in vitro for potential plant colonization ability, antifungal activity, and multiple plant growth-promoting mechanisms. These traits were integrated into composite indices and analyzed using multivariate approaches to guide the selection of promising isolates. Selected candidates were subsequently evaluated in vivo for biocontrol and plant growth at both seedling and productive stages, and most isolates exhibited consistent effects. Isolates from the genera *Stenotrophomonas*, *Pseudomonas*, and *Bacillus* reduced fungal disease incidence to 2–9% (control disease: 80%). A *Bacillus* isolate increased seedling biomass by 54% in lettuce and 38% in tomato. Under productive conditions, lettuce marketable weight increased by 21–37%, whereas tomato yield showed positive but non-significant increases (~21–25%) after inoculation with *Pseudomonas* or *Bacillus* isolates. Overall, this work provides a structured framework for PGPB bioprospecting and validation, combining laboratory screening, composite indices, multivariate analyses, and multi-stage in vivo assays under realistic horticultural conditions.

Keywords: bioprospecting; plant growth-promoting bacteria; biocontrol; endophytic bacteria; sustainable horticulture; lettuce; tomato



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1. Introduction

Tomato (*Solanum lycopersicum* L.) production is one of the most important horticultural crops worldwide [1] due to its high economic value, nutritional relevance, and widespread demand for both fresh and processed products. In Argentina, tomato is among the most economically and socially significant vegetable crops, supplying both fresh markets and the agroindustry, with an annual production of approximately 1.4 million tons, which positions the country as one of the leading producers in South America [2]. Production for

fresh consumption accounts for approximately 60–70% of the total output and is mainly concentrated in peri-urban areas, such as the Cinturón Hortícola Platense (CHP), a major horticultural hub surrounding the city of La Plata, Buenos Aires [3]. Tomato cultivation in this region is conducted almost entirely under greenhouse conditions, allowing for more efficient crop management and the implementation of double annual cycles, thereby maximizing land use and installed infrastructure. Despite these advantages, the system faces several challenges related to productive sustainability, including pest and disease pressure, high dependence on chemical inputs, and social issues associated with horticultural labor [4]. In this context, the adoption of agroecological practices and the development of bioinputs emerge as key strategies to enhance system resilience and sustain regional competitiveness. Among the most promising approaches is the use of plant growth-promoting bacteria (PGPB), a diverse group of microorganisms capable of enhancing plant development through direct mechanisms—such as biological nitrogen fixation, phosphorus solubilization, and phytohormone production—as well as indirect mechanisms, including the induction of systemic resistance and the biological control of phytopathogens [5]. Within this group, endophytic bacteria present distinct ecological advantages, making them particularly attractive candidates for the development of novel bioinput formulations. By inhabiting internal plant tissues, endophytes establish intimate and stable associations with the host, enabling direct interaction with plant metabolism and localized delivery of bioactive compounds. Compared with soil- and rhizosphere-associated microorganisms, they are exposed to reduced microbial competition and environmental fluctuations, which may favor persistence and functional stability within the plant host [6,7]. Accordingly, characterizing PGPB associated with tomato plants in the CHP is essential for identifying locally adapted strains with high biotechnological potential, maximizing their effectiveness, improving the reproducibility of growth-promoting effects, and increasing the likelihood of successful field application.

Despite the extensive body of literature demonstrating the potential of PGPB to enhance crop productivity and sustainability, their effective translation into reliable and reproducible agricultural bioinputs remains limited. A major constraint lies in the lack of structured, standardized, and field-relevant frameworks that integrate microbial isolation, functional characterization, and *in vivo* validation. Many studies rely on isolated *in vitro* traits or short-term assays, which often perform inconsistently under productive conditions, resulting in variable responses and limited reproducibility across environments. Recent reviews have highlighted the need for more integrative and quantitative bioprospecting strategies capable of prioritizing microbial candidates with true agronomic potential, ecological competence, and applicability at scale [8,9]. In this context, the development and validation of systematic methodological approaches represent a critical step toward bridging the gap between microbial discovery and the development of effective agricultural bioinputs.

Against this background, this study aimed to characterize culturable bacteria associated with tomato plants (*Solanum lycopersicum* var. Elpida) grown under greenhouse conditions in the CHP, with particular emphasis on endophytic taxa. A sequential and integrative approach was applied, combining ecological origin, *in vitro* functional traits related to biocontrol, plant colonization, and growth promotion, the construction of quantitative composite indices, and multivariate analyses to support isolate selection. Promising candidates were subsequently validated *in vivo* through plant growth-promotion assays in lettuce and tomato at both seedling and productive stages. This framework enables the objective identification of bacterial isolates with agronomic relevance and provides a robust basis for the development of effective agricultural bioinputs under realistic horticultural conditions.

2. Materials and Methods

2.1. Experimental Sites and Sample Collection

Sampling was conducted in tomato crops grown in two commercial greenhouses within the CHP (−57.9344, −35.0158; and −57.9593, −35.0207). At both sites, tomato plants (*Solanum lycopersicum* L.) var. Elpida (Enza Zaden) were transplanted in early January 2016 following standard agronomical practices. Three composite samples of bulk soil (BS), rhizosphere soil (Rh), and plant tissues—roots (Rt), stems (St), and fruits (Fr)—were collected from tomato crops in each greenhouse in May 2016, at the fourth truss harvest stage. Bulk soil was obtained by pooling three subsamples collected from nine randomly selected inter-plant points per site (0–10 cm depth). Rhizosphere soil was collected from nine randomly selected plants per site by shaking roots to detach adhering soil, pooling subsamples from three plants into one composite. Roots, stems, and fruits from the same plants were grouped into three sets of three plants each to generate composite Rt, St, and Fr samples per site. All samples were collected in sterile flasks, transported on ice, and processed within 24 h at MAAlab (CINDEFI, La Plata, Argentina). In total, 30 composite samples were analyzed, corresponding to 5 microhabitats with 3 independent composite samples per microhabitat collected at each of two greenhouse sites (5 microhabitats × 3 replicates × 2 sites). The two greenhouse sites were not considered independent experimental replicates but were sampled to broaden the recovery of cultivable bacterial diversity under comparable agronomic conditions. Soil physicochemical properties were determined using standardized procedures (see Table S2.1.1 in S2. Supplementary data supporting Results). Detailed experimental conditions for all experiments described in the sections below, including protocols and assay-specific parameters, are provided in Supplementary Material S1.

2.2. Isolation, Enumeration, and Preservation of Cultivable Bacteria

From each composite sample of BS (10 g) and Rh (3 g), suspensions were prepared in 100 mL of PBS buffer containing 0.1% (*w/w*) Tween 80 and shaken at 150 rpm for 40 min, following a modified version of the protocol described by [10]. Serial decimal dilutions (10^{-1} to 10^{-6}) were prepared, and 100 μ L aliquots were plated in triplicate on Reasoner's 2A medium (R2A; composition per liter: 0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g casein hydrolysate, 0.5 g glucose, 0.5 g starch, 0.3 g K_2HPO_4 , 0.3 g sodium pyruvate, 0.024 g $MgSO_4 \cdot 7H_2O$, and 15 g Agar; pH regulated at 6.5 with H_3PO_4 20% *v/v*) for the isolation of heterotrophic bacteria. After incubation at 30 °C for 72 h in darkness, the number of colony-forming units (CFUs) per gram of sample was calculated.

Root (Rt), stem (St), and fruit (Fr) tissues were processed to obtain endophytic bacterial isolates following a modified surface-disinfection protocol described by [11,12]. Briefly, tissues were washed, surface-disinfected using NaClO or ethanol depending on tissue type, and rinsed with sterile distilled water. The effectiveness of surface disinfection was verified by plating the final rinse water and imprinting disinfected tissues onto R2A medium; the absence of growth after 48 h confirmed the removal of epiphytic bacteria. Disinfected tissues were aseptically macerated, serially diluted, and plated on R2A medium in triplicate. Plates were incubated at 30 °C for 48 h in darkness, and CFU counts were used to estimate endophytic bacterial populations.

Distinct colony morphotypes, defined by differences in color, shape, texture, margin, and elevation, were selected directly from the R2A plates used for CFU enumeration. Within each microhabitat, all visibly distinct colony morphologies observed across the corresponding plates were isolated in order to capture the widest possible culturable bacterial diversity associated with that specific compartment. This selection was performed independently for each microhabitat; therefore, similar morphotypes could be recovered

from different compartments. Isolates were purified by repeated streaking and stored in 20% (v/v) glycerol at -80°C .

2.3. *In Vitro* Characterization of Bacterial Isolates for Plant Colonization Potential

The bacterial isolates were assessed for their potential to colonize plant tissues through *in vitro* assays evaluating the production of extracellular lytic enzymes and the ability to form biofilms. Based on these traits, two indices were calculated: the Enzymatic Characterization Index for Plant Colonization (ECi) and the Biofilm Index (Bf), following an integrative and comparative approach conceptually adapted from the indices proposed by [13], and tailored to the traits evaluated in this study. These indices were subsequently integrated into a Plant Colonization Index (PCi) as an overall, non-predictive metric designed to rank isolates according to their relative colonization potential. All indices used in this study represent standardized summaries of experimentally measured traits, rely exclusively on normalized experimental data, and were applied uniformly across all isolates. Their role was strictly restricted to internal ranking and candidate prioritization within the analyzed collection. The same conceptual basis and scope apply to the composite indices described in the subsequent sections addressing biocontrol and plant growth-promotion potential.

2.3.1. Lytic Enzyme Activity and ECi Calculation

Cellulase and pectinase production were evaluated qualitatively on specific agar media as described by [14,15] and standard protocols. A positive result was recorded when isolates produced a clear halo surrounding the colony, indicating enzymatic hydrolysis of the specific substrate. Each determination was scored as either positive (1) or negative (0). The ECi for each isolate was calculated as:

$$\text{ECi}_j = \sum \text{EC}_{jk} / n$$

where EC represents the binary result of isolate j for enzyme assay k , and n the number of tested enzymatic activities. When an enzymatic activity could not be determined due to a lack of growth on the corresponding medium, it was recorded as not determined (nd) and excluded from both the numerator and denominator of the ECi calculation.

2.3.2. Biofilm Formation and Bf Calculation

Biofilm formation was quantified in sterile 96-well polystyrene microplates using the method described by [16]. Absorbance at 590 nm was measured, and isolates were classified into four categories from non-producer to strong producer according to the thresholds described by [17]. Each isolate was assigned a numerical value (Bf) ranging from 0 (no producer) to 1 (strong producer).

2.3.3. Plant Colonization Index (PCi)

The potential colonization capacity of each isolate was expressed as the Plant Colonization Index (PCi), integrating both enzymatic activity and biofilm formation:

$$\text{PCi}_j = (\text{ECi}_j + \text{Bf}_j) / 2$$

The PCi values ranged from 0 (absence of lytic enzymes and no biofilm formation) to 1 (positive for both enzymatic activities and strong biofilm formation).

2.4. *In Vitro* and *In Vivo* Characterization of Bacterial Isolates for Antagonistic Potential

Bacterial isolates were characterized for their antagonistic potential against phytopathogenic fungi through a combination of *in vitro* and *in vivo* assays. *In vitro* eval-

uations included the assessment of extracellular lytic enzyme production and dual-culture assays against four major fungal pathogens of horticultural relevance—*Alternaria alternata* (#25031, IMyZA collection, La Plata, Argentina), *Botrytis cinerea*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* (strains LPSC-8, LPSC-1017, and LPS-28138, respectively, from Carlos Spegazzini Institute, La Plata, Argentina)—allowing the quantification of fungal growth inhibition. These traits were integrated into composite indices to estimate the antagonistic capacity of each isolate, and combined with plant colonization ability into a Biocontrol Potential Index (BPI). Based on index values, selected isolates were further evaluated for conidial germination inhibition and in vivo assays using lettuce as a model system.

2.4.1. Extracellular Lytic Enzyme Production and ECiA Calculation

The production of extracellular lytic enzymes associated with antagonistic activity was evaluated qualitatively for each isolate [18], including proteases, chitinases, lipases, and amylases, following standard procedures [19–21]. Enzymatic assays were performed in duplicate, and enzymatic activity was recorded as positive or negative based on the presence of clear hydrolysis zones around bacterial colonies.

An Enzymatic Characterization Index for Antagonism (ECiA) was calculated for each isolate based on binary scoring of enzymatic activities (positive = 1; negative = 0):

$$ECiA_j = \sum ECA_{jk} / n$$

where ECA represents the binary outcome of isolate j for enzymatic assay k , and n corresponds to the number of enzymatic activities evaluated. When an activity could not be determined, it was recorded as not determined (nd) and excluded from both the numerator and denominator of the ECiA calculation.

2.4.2. Dual Culture Assays and PIRG Determination

Antagonistic activity against phytopathogenic fungi was evaluated using dual culture assays on potato dextrose agar (PDA), as described by [22]. Bacterial isolates were initially screened in multi-isolate plates, and those showing inhibitory effects were subsequently tested individually against each pathogen. Antagonism was quantified as the Percentage of Inhibition of Radial Growth (PIRG), calculated by comparing fungal growth in the presence of bacteria with that in control plates:

$$PIRG_{ij} = (H_i \text{ control} - H_{ij}) / H_i \text{ control}$$

where H_{ij} represents the radial growth diameter (mm) of phytopathogen i in the presence of bacterial isolate j , and H_i control corresponds to the radial growth diameter of phytopathogen i on control plates without bacterial inoculation. A PIRG value equal to or greater than 20% was considered indicative of antagonistic activity. A PIRG value $\geq 20\%$ was considered indicative of antagonistic activity.

2.4.3. Antagonistic Potential Index (APi) and Biocontrol Potential Index (BPI) and Selection of Promising Biocontrol Isolates

To integrate the different antagonistic traits evaluated, two composite indices were calculated. The Antagonistic Potential Index (APi) combined extracellular enzymatic activity related to antagonism (ECiA) and the mean inhibition of fungal growth across all tested phytopathogens ($PIRG_{av}$):

$$APi = [ECiA + (2 \times PIRG_{av})] / 2$$

PIRG was weighted more heavily than enzymatic activity because it reflects the effective inhibition of fungal growth, providing a direct functional measure of antagonism rather than a proxy of potential mechanisms.

In addition, a Biocontrol Potential Index (BPi) was calculated by integrating APi with the PCi, providing an overall estimate of each isolate's potential as a biocontrol agent:

$$\text{BPi} = (\text{PCi} + \text{APi}) / 2.$$

Based on PIRG and BPi, isolates were selected for further testing: (i) the five isolates with the highest PIRG for each phytopathogen were designated as pathogen-specific antagonists, and (ii) the five isolates with the highest BPi values were designated as generalist biocontrol agents for all tested fungi.

2.4.4. Inhibition of Conidial Germination

Selected isolates were further evaluated for their ability to inhibit conidial germination of three phytopathogenic fungi (*Alternaria alternata*, *Botrytis cinerea*, and *Fusarium oxysporum*), following the protocol recommended by [23]. For each phytopathogen, both isolates previously selected as pathogen-specific antagonists and those selected as general biocontrol candidates were included in the assay. Conidial suspensions and bacterial cultures were standardized to an optical density of 0.2 and mixed in equal volumes in sterile tubes, in triplicate. Germination was assessed after 24, 48, 72, and 168 h of incubation. At each time point and for each treatment, 100 conidia were microscopically examined, and the number of germinated conidia was recorded. Conidia were considered germinated when the germ tube length equaled or exceeded the conidium length.

2.4.5. In Vivo Biocontrol on Lettuce Seedlings

The in vivo biocontrol potential of selected bacterial isolates was evaluated in a seed germination assay, using lettuce (*Lactuca sativa* var. *sagess*) as a plant model. Based on preliminary assays, this evaluation was conducted exclusively with *Botrytis cinerea*, as no visible disease symptoms were observed in control treatments inoculated with the other phytopathogens used. In triplicate, lettuce seeds were surface-disinfected and inoculated with *Botrytis cinerea* conidia, and subsequently treated individually with each bacterial suspension, both standardized to an optical density of 0.2. A negative control consisted of seeds treated only with sterile distilled water, whereas a positive control included seeds inoculated exclusively with the fungal conidial suspension. Seeds were incubated under controlled conditions, and germination and disease development were assessed five days after inoculation. Germination percentage was determined by counting germinated seeds out of 100 seeds per treatment. Disease incidence was calculated as the proportion of seedlings showing disease symptoms relative to the total number of seedlings, and disease severity was scored using four categories ranging from healthy to dead seedlings.

2.5. In Vitro and In Vivo Characterization of Bacterial Isolates for Plant Growth-Promotion Potential

Bacterial isolates were characterized for their plant growth-promoting (PGP) potential through a set of in vitro assays evaluating phosphate solubilization, siderophore production, auxin synthesis, and biological nitrogen fixation. These functional traits were integrated into a Plant Growth-Promoting Trait Index (PGPi) to allow comparative assessment across the bacterial collection. In addition, PGPi values were combined with plant colonization ability into an overall Plant Growth-Promotion Potential Index (PGPPi). Based on index values, selected isolates were further evaluated in vivo for their capacity to promote plant growth under controlled and production conditions.

2.5.1. Phosphate Solubilization

Phosphate solubilization of bacterial isolates was evaluated using NBRIP agar supplemented with tricalcium phosphate, following [24]. Solubilization activity was qualitatively assessed based on the presence and relative size of clear halos surrounding bacterial colonies and classified into three categories: (+) halo coincident with the colony edge; (++) halo slightly larger than the colony; and (+++) halo much larger than the colony.

Isolates showing moderate to strong activity were further evaluated quantitatively, in duplicate, by measuring colony diameter (D_c) and phosphate-solubilization halo diameter (D_h), to calculate Phosphate Solubilization Efficiency (PSE) as [25]:

$$PSE_j = (D_{hj} - D_{cj})/D_{cj}$$

To allow comparison among isolates, PSE values were normalized between 0 and 1, generating a Phosphate Solubilization Index (PSi):

$$PSi_j = PSE_j / PSE_{\max}$$

Isolates showing only qualitative activity (– and +) were assigned a fixed low PSE value (0.0 and 0.10, respectively) for further index calculation.

2.5.2. Siderophore Production

Siderophore production was evaluated qualitatively using a plate overlay technique with a Chrome Azurol S indicator assay [26]. Each assay was performed in duplicate. The presence of yellow halos surrounding bacterial colonies was recorded as indicative of siderophore secretion and classified into three intensity levels based on the diameter of the halo: weak (+), moderate (++), or strong (+++). Isolates showing no visible halo were considered negative. For comparative purposes and further analysis, qualitative scores were converted into a numeric siderophore production score (SID): + = 0.33, ++ = 0.66, +++ = 1.00, and negative = 0, which was used for subsequent index calculations.

2.5.3. Biological Nitrogen Fixation

The potential for biological nitrogen fixation (BNF) was assessed indirectly by evaluating growth in nitrogen-free semi-solid media (LGI and NFb) according to [27] in bacterial endophytes only. Isolates were spike-inoculated in duplicate and incubated in darkness at 30 °C for 10 days. The formation of a characteristic subsurface pellicle in at least one of the media was scored as positive (BNF = 1), whereas the absence of growth in both media was scored as negative (BNF = 0), and used for subsequent index calculations.

2.5.4. Auxin Production

Auxin production by bacterial isolates was evaluated qualitatively using a colorimetric assay based on Salkowski's reagent [28]. Isolates were grown in DF medium supplemented with L-tryptophan, and auxin production was inferred from the development of a pink coloration after reaction with the reagent. Based on absorbance intensity at 590 nm, isolates were classified into four categories: non-producer (–), low (+), moderate (++), and high (+++) auxin producers, subsequently converted into normalized auxin production scores (AUX) of 0, 0.33, 0.67, and 1.0, respectively, for use in plant growth-promotion index calculations.

2.5.5. Calculation of Plant Growth-Promotion Indices and Selection of Promising Isolates

A Plant Growth-Promoting Trait Index (PGPi) was calculated for each isolate by integrating the normalized scores of individual *in vitro* traits:

$$\text{PGPi}_j = \text{PSi}_j + \text{SID}_j + \text{BNF}_j + \text{AUX}_j$$

where PSi, SID, BNF, and AUX correspond to the normalized indices of phosphate solubilization, siderophore production, biological nitrogen fixation, and auxin production, respectively.

To obtain an overall measure of plant growth-promotion capacity, the Plant Growth-Promotion Potential Index (PGPPi) was calculated by integrating PGPi with the Plant Colonization Index (PCi):

$$\text{PGPPi}_j = [\text{PCi}_j + (2 \times \text{PGPi}_j)]/2$$

Greater weight was assigned to PGPi, as the expression of plant growth-promotion mechanisms represents the primary driver of growth enhancement, whereas effective colonization constitutes a necessary but complementary prerequisite for the manifestation of these effects.

Based on PGPPi values, the ten highest-ranked isolates were selected as the most promising candidates for subsequent *in vivo* plant growth-promotion assays.

2.5.6. In Vivo Plant Growth-Promotion Assays

In vivo plant growth-promotion assays were conducted to evaluate the effects of selected bacterial isolates on lettuce and tomato at both the seedling and productive stages, under controlled and production-relevant conditions, following protocols described by [11,29,30].

At the seedling stage, assays were first performed under controlled growth-chamber conditions and subsequently validated in a commercial nursery system. Bacterial suspensions were applied at sowing, and plant growth parameters were evaluated after 30 days, including root and shoot dry biomass, and leaf area by imaging recorder and Image J v.1.53e software [31].

To assess performance under productive conditions, seedlings were transplanted to experimental field plots managed according to commercial horticultural practices. Lettuce and tomato plants were reinoculated before transplanting and evaluated throughout the production cycle using randomized complete block designs. In lettuce, commercial yield was assessed at harvest, whereas in tomato, fruit number, fruit weight, and total yield per plant were recorded over the production period.

2.6. Taxonomic Assignment and Phylogenetic Analysis of Endophytic Bacteria

Taxonomic identification was performed for all endophytic bacterial isolates recovered from Rt, St, and Fr tissues, as well as for those isolates selected for *in vivo* bio-control and plant growth-promotion assays. Genomic DNA was extracted from single bacterial colonies using a boiling lysis method. Partial amplification of the 16S rRNA gene was carried out using primers 27F (3'-AGAGTTTGATCMTGGCTCAG-5') and 907R (3'-CCGTCAATTCMTTTRAGTTT-5'), targeting the V3–V7 hypervariable regions. PCR products were sequenced by Macrogen Inc. (Seoul, Republic of Korea). Raw sequences were quality-checked and edited using ApE v3.1.3 [32] and compared against the NCBI nucleotide database using BLASTn v2.13. Isolates were assigned to the closest taxonomic affiliation based on sequence similarity and percentage identity.

2.7. Statistical Data Analysis

Infostat software v.2018 [33] was used for statistical analysis on data. A one-way analysis of variance (ANOVA) was performed to evaluate differences in bacterial population density, expressed as CFU g⁻¹, among microhabitats. Prior to analysis, CFU data were log-transformed to meet the assumptions of homoscedasticity and normality. Microhabitat was considered the single fixed factor, with five levels: bulk soil (BS), rhizosphere soil (Rh), root endosphere (Rt), stem endosphere (St), and fruit endosphere (Fr). For each microhabitat, six independent observations were analyzed ($n = 6$), resulting in a total sample size of 30 ($N = 30$).

Multivariate analysis was performed to integrate antagonism-, colonization-, and biocontrol-related traits: PIRG values against individual phytopathogens, together with ECiA, APi, PCi, and BPi, were included for each isolate. Prior to principal component analysis (PCA), variables were standardized and the correlation structure among variables was examined to confirm the suitability of the dataset for multivariate analysis. PCA was conducted on the correlation matrix without rotation, and PCA biplots were generated at the isolate and microhabitat levels. Conidial germination data were analyzed independently for each phytopathogen using a generalized linear mixed model (binomial distribution, logit link), with bacterial treatment (each phytopathogen combined with each selected bacterial isolate, including a control without bacteria) and incubation time (24, 48, 72, and 168 h) as fixed factors. Replicate was included as a random effect to account for repeated measurements over time. When required, models were checked for overdispersion and adjusted accordingly. Disease incidence in lettuce seedlings was analyzed using a one-way ANOVA, where bacterial treatment (*B. cinerea* combined with each selected bacterial isolate) was considered the experimental factor, including a negative control (uninoculated seeds) and a positive control (seeds inoculated only with *B. cinerea*). When significant effects were detected, comparisons of means were conducted using Tukey's post hoc test and $p < 0.05$ were considered statistically significant.

Multivariate analysis was performed to integrate plant growth-promotion and colonization-related traits: PSi values together with SID, BNF, AUX, PCi, and PGPPi were included for each isolate. PCA was performed under the same standardization and analytical criteria described above, and biplots were generated at the isolate and microhabitat levels. For both lettuce and tomato, and for the seedling and productive stages evaluated, plant growth and yield parameters—including root, shoot, and total biomass accumulation, leaf area, commercial fresh weight, tomato fruit yield per plant (total weight and fruit number), and individual fruit weight—were analyzed by ANOVA. A randomized complete single-factor design was used for seedling-stage assays, whereas a randomized complete block design was applied for productive-stage assays, with bacterial treatment as the main experimental factor (11 levels: ten selected bacterial isolates plus an uninoculated control) and block (row) included as a blocking factor when applicable. When the bacterial treatment factor showed significant effects ($p < 0.05$), comparisons of means were conducted using Dunnett's post hoc test and $p < 0.05$ were considered statistically significant.

In all parametric statistical tests applied, the assumptions of normality and homoscedasticity of residuals were confirmed (95% confidence) with the modified Shapiro–Wilks and Cochran tests, respectively.

3. Results

3.1. Enumeration, Isolation, and Taxonomic Characterization of Cultivable Bacteria

Cultivable bacteria were successfully recovered from all sampled microhabitats using the applied isolation protocols. The highest bacterial abundances were observed in rhizosphere (Rh) and bulk soil (BS) samples, with population densities reaching 10⁹–10¹⁰ CFU

g^{-1} and $\sim 10^7 \text{ CFU g}^{-1}$, respectively (Figure 1A). Bacterial growth was also consistently detected following maceration of surface-disinfected plant tissues, confirming the presence of endophytic bacterial populations. Endophytic densities decreased progressively across plant compartments, from approximately 10^6 CFU g^{-1} in roots (Rt) to $<10^3 \text{ CFU g}^{-1}$ in fruits (Fr). No bacterial growth was detected on control R2A plates after tissue disinfection, demonstrating the effectiveness of the procedure and confirming that recovered colonies corresponded to internal endophytic bacteria. Statistical analysis indicated that bacterial abundance was significantly influenced by microhabitat type ($p < 0.01$), with all evaluated microhabitats differing significantly from each other.

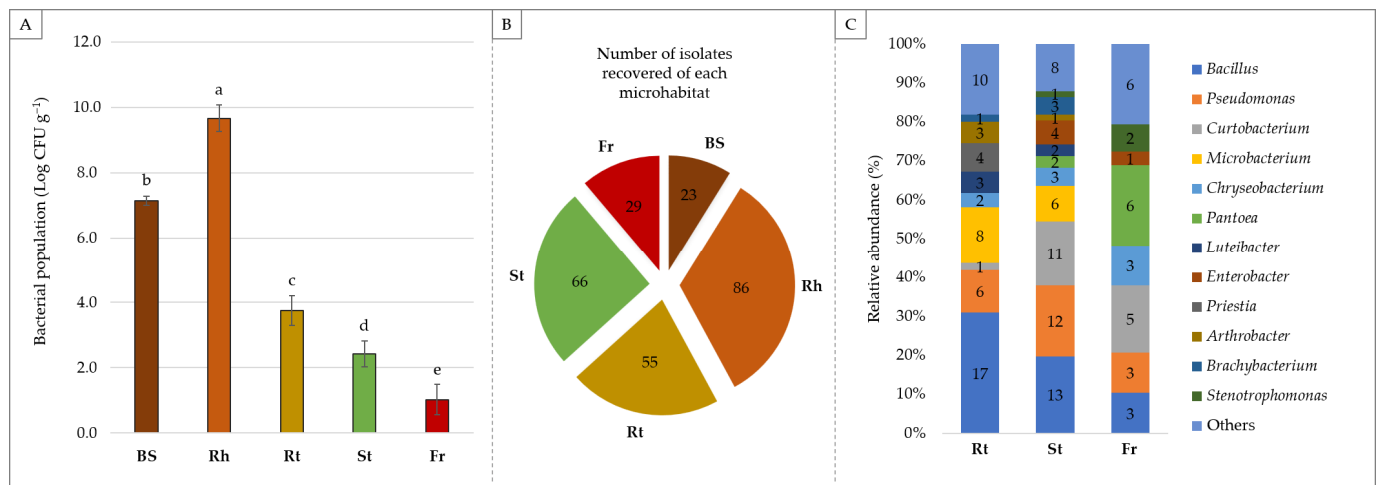


Figure 1. Cultivable bacterial abundance and isolate recovery across different microhabitats associated with tomato crops. (A) Bacterial population densities expressed as log CFU g⁻¹ (mean $\pm$ SE) in bulk soil (BS), rhizosphere soil (Rh), root endosphere (Rt), stem endosphere (St), and fruit endosphere (Fr). Different letters indicate significant differences among microhabitats according to one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). (B) Number of bacterial isolates selected based on distinct colony morphotypes recovered from each microhabitat. (C) Relative taxonomic composition of endophytic bacterial isolates at the genus level across Rt, St, and Fr.

A total of 259 bacterial isolates were selected based on distinct colony morphologies from R2A plates across all sampled microhabitats. Detailed information on the isolate collection, including microhabitat of origin, in vitro functional characterization, calculated indices, and taxonomic assignment of endophytic isolates, is provided in Supplementary Material S3. The highest number of isolates was recovered from the Rh, followed by the St and Rt, whereas BS and the Fr contributed comparatively fewer isolates to the collection (Figure 1B).

Taxonomic assignment was performed exclusively for endophytic isolates and revealed their distribution across four major bacterial phyla: *Proteobacteria* (37.5%), *Actinobacteria* (34.0%), *Firmicutes* (19.3%), and *Bacteroidetes* (9.2%). In total, 33 bacterial genera were identified. The most frequently represented genera were *Bacillus*, *Pseudomonas*, *Curtobacterium*, *Microbacterium*, *Chryseobacterium*, and *Pantoea*. Genus richness varied among plant organs, with 18 genera detected in Rt, 20 in St, and 14 in Fr. Relative abundance profiles (Figure 1C) showed that *Bacillus* and *Pseudomonas* dominated Rt, St and Fr; however, St exhibited a more diverse taxonomic composition, including a higher representation of *Curtobacterium* and *Microbacterium*.

3.2. In Vitro Characterization of Plant Colonization Potential

The characterization of colonization-related traits showed that both lytic enzymatic activities and biofilm formation were present across the tomato-associated bacterial collec-

tion. Isolates producing cellulase and/or pectinase were mainly recovered from the Rh, with additional representatives detected in BS and plant endospheres (Figure 2).

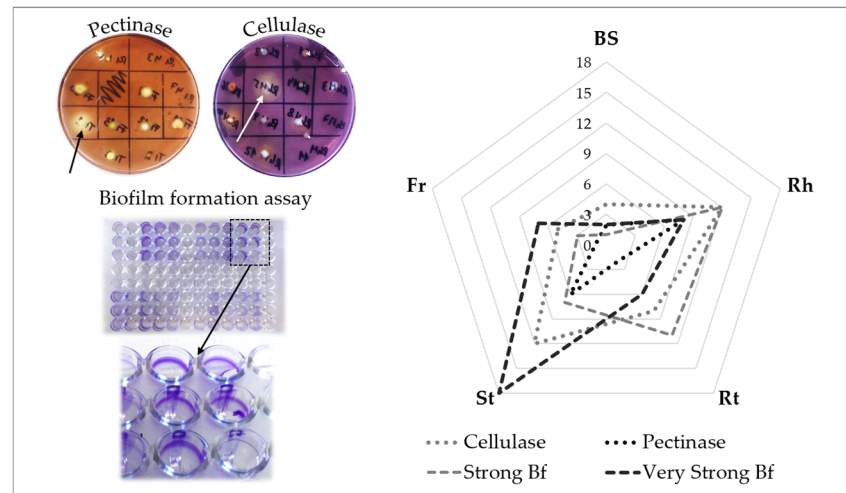


Figure 2. Distribution of colonization-related traits among bacterial isolates recovered from different tomato-associated microhabitats. The radar plot shows the number of isolates positive for cellulase, pectinase, and strong or very strong biofilm formation in bulk soil (BS), rhizosphere (Rh), root (Rt), stem (St), and fruit (Fr) endospheres. Values correspond to absolute counts of positive isolates per microhabitat. Representative images of positive enzymatic activity, indicated by arrows in the figure, and biofilm formation assays are shown.

Biofilm formation capacity varied widely among isolates. Approximately half of the collection exhibited null or weak biofilm formation, whereas the remaining isolates were classified as moderate to very strong producers. Isolates displaying the highest biofilm-forming capacity were predominantly recovered from plant-associated microhabitats, particularly Rt and St (Figure 2).

Integration of enzymatic activity and biofilm formation into the Plant Colonization Index (PCi) allowed comparison of colonization-related traits across microhabitats. When PCi values were averaged by microhabitat, plant-associated compartments consistently showed higher mean PCi values than BS, reflecting a higher proportion of isolates displaying positive colonization-related traits (see Table S2.2.1 in S2. Supplementary data supporting Results).

Based on PCi values, a subset of isolates with high colonization potential was identified. In total, 12 isolates reached PCi values ≥ 0.75 , all originating from plant-associated microhabitats. These high-scoring isolates were mainly affiliated with the genera *Bacillus* (4 isolates), *Pseudomonas* (2), *Flavobacterium* (1), and *Enterobacter* (1), while the remaining four isolates could not be taxonomically resolved at the genus level (see Table S2.2.2 in S2. Supplementary data supporting Results). All high-PCi isolates combined strong biofilm-forming capacity with at least one lytic enzymatic activity.

3.3. In Vitro and In Vivo Characterization of Antagonistic Potential

Bacterial isolates were screened for extracellular enzymatic activities associated with antagonistic potential, including amylase, protease, chitinase, and lipase production. Overall, 40.9% of the isolates displayed at least one enzymatic activity, indicating a high degree of functional diversity related to antagonistic traits within the bacterial collection (Figure 3A). Protease and lipase were the most frequently detected activities, whereas chitinase showed the lowest occurrence. Chitinase-positive isolates were predominantly associated with the Rh and Rt. The distribution of enzymatic activities varied among plant microhabitats,

with Rh- and Rt-derived isolates exhibiting the highest proportion of enzymatically active strains. At the genus level, *Bacillus* and *Pseudomonas* accounted for the majority of enzymatic activities, particularly in amylase- and protease-positive isolates. *Enterobacter* and *Serratia* strains were predominantly associated with lipase and chitinase production.

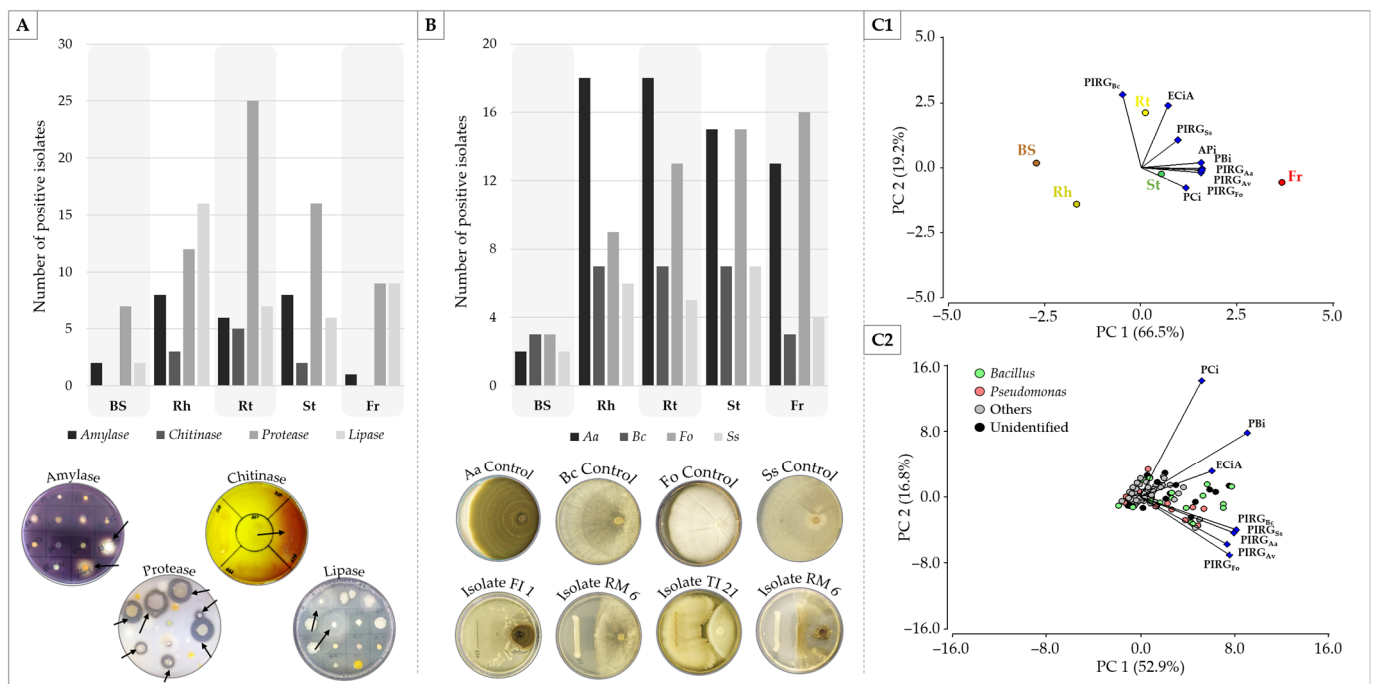


Figure 3. Summary of in vitro characterization of antagonistic potential of bacterial isolates according to the microhabitat of origin. (A) Number of isolates positive for extracellular enzymatic activities associated with antagonism, grouped by microhabitat (BS, bulk soil; Rh, rhizosphere; Rt, root endosphere; St, stem endosphere; Fr, fruit endosphere). Representative plate assays are shown below, where positive enzymatic activity is indicated by black arrows. (B) Number of isolates exhibiting antagonistic activity in dual-culture assays against *Alternaria alternata* (Aa), *Botrytis cinerea* (Bc), *Fusarium oxysporum* (Fo), and *Sclerotinia sclerotiorum* (Ss), grouped by microhabitat (BS, bulk soil; Rh, rhizosphere; Rt, root endosphere; St, stem endosphere; Fr, fruit endosphere), with representative plates examples of growth inhibition. (C) Principal component analysis (PCA) at the microhabitat (C1) and isolate (C2) levels, using percentage of inhibition of radial growth (PIRG) values against individual phytopathogens, together with the Enzymatic Characterization Index for Antagonism (ECiA), Antagonistic Potential Index (APi), Plant Colonization Index (PCi), and Biocontrol Potential Index (BPi) as variables.

All bacterial isolates were evaluated in vitro for antagonistic activity against four phytopathogenic fungi—*Alternaria alternata* (Aa), *Botrytis cinerea* (Bc), *Fusarium oxysporum* (Fo), and *Sclerotinia sclerotiorum* (Ss) (Figure 3B). Overall, 91 isolates (35.1% of the collection) inhibited the radial growth of at least one phytopathogen (PIRG > 20%): the percentage of inhibition of radial growth (PIRG) ranged from zero up to ~90% in the most effective isolates. Antagonistic activity was most frequently observed against Aa (66 isolates) and Fo (56 isolates), followed by Bc (27 isolates) and Ss (24 isolates). The distribution of antagonistic isolates by microhabitat and target pathogen is summarized in Figure 3B. The majority of antagonistic isolates (>60%) originated from plant-associated microhabitats (Rt, St, or Fr), whereas BS-derived isolates showed the lowest frequency of antagonistic activity. Most antagonistic isolates were active against a single pathogen, whereas 14 isolates exhibited broad-spectrum antagonism, inhibiting all four fungi (see Table S2.3.1 in S2. Supplementary data supporting Results). Taxonomic assignment indicated that antagonistic activity was mainly associated with the genera *Bacillus*, *Pseudomonas*, *Chryseobacterium*,

and *Pantoea*. Among these, *Bacillus*, *Pseudomonas*, and *Pantoea* included isolates exhibiting broad-spectrum antagonism.

Integration of PIRG values with ECiA into the Antagonistic Potential Index (APi) enabled a comparative assessment of overall antagonistic performance among isolates. Antagonistic performance (APi) was further combined with colonization capacity (PCi) to calculate the Biocontrol Potential Index (BPi).

Principal component analysis (PCA) was used to explore multivariate patterns among antagonism- and colonization-related indices at both the microhabitat and isolate levels. The correlation matrix, together with eigenvalues and the percentage of variance explained by each principal component, is presented in Supplementary Material S2. In the microhabitat-based PCA (Figure 3(C1)), the first two components explained 66.5% (PC1) and 21.9% (PC2) of the total variance, accounting for 88.4% of the cumulative variance and supporting the interpretation of the PCA biplots. Plant-associated microhabitats, particularly Rt, St, and Fr, were positioned toward the positive side of PC1 and were closely associated with higher values of antagonism- and biocontrol-related indices. In contrast, BS clustered toward the negative region of PC1, reflecting lower overall antagonistic potential. Consistently, averaging index values by microhabitat also revealed higher mean values in all plant-associated compartments compared to BS (see Table S2.3.6 in S2, Supplementary data supporting Results), supporting the PCA-based differentiation. In the isolate-based PCA (Figure 3(C2)), the first two principal components explained a substantial proportion of the total variance (PC1: 52.9% and PC2: 16.8%, accounting for 69.7% of the cumulative variance). PC1 was mainly driven by antagonism-related variables, including PIRG values against the different phytopathogens as well as the integrated APi and BPi, whereas PC2 showed a stronger association with colonization-related traits, particularly PCi. Most isolates clustered around the origin, indicating moderate or low index values; however, a subset of isolates clearly separated along the positive axis of PC1 and/or PC2, reflecting high antagonistic performance and/or strong colonization capacity. Notably, many of these isolates were affiliated with the genera *Bacillus* and *Pseudomonas*, which are well known for their biocontrol potential. Based on these patterns, isolates were selected for downstream biocontrol assays using two complementary criteria. For each phytopathogen, the five isolates displaying the highest PIRG values were selected as pathogen-specific antagonists. In addition, the five isolates with the highest Biocontrol Potential Index (BPi) were selected as general biocontrol candidates. The selected isolates largely corresponded to those separating from the central PCA cluster in panel C2 and are summarized in Table 1.

The ability of selected bacterial isolates to inhibit conidial germination was evaluated in vitro against *Botrytis cinerea*, *Alternaria alternata*, and *Fusarium oxysporum*. For *B. cinerea*, the model revealed significant effects of isolate-treatment, incubation time, and a significant interaction ($p < 0.01$ in all cases, Table S2.3.7 in S2), indicating that the inhibitory effect of bacterial isolates on conidial germination was strain-dependent and varied over time. In the absence of bacterial antagonists, conidial germination increased steadily from 24 to 168 h. In contrast, the presence of bacterial isolates strongly reduced germination throughout the incubation period, with marked differences in the temporal dynamics among strains. Several isolates caused an early and sustained inhibition of germ tube emergence, maintaining very low germination probabilities across all evaluation times, whereas others showed weaker or delayed inhibitory effects.

Table 1. Bacterial isolates selected as pathogen-specific and general biocontrol candidates based on in vitro antagonistic performance and biocontrol-related indices (PIRG and PBi).

Isolate	Microhabitat	Taxonomy	Pathogen-Specific Antagonists				General Antagonists (PBi)
			PIRG _{Aa}	PIRG _{Bc}	PIRG _{Fo}	PIRG _{Ss}	
RhM 38	Rh	nd				0.50	0.90
RhI 22		nd					0.76
RhI 5		<i>Pseudomonas</i>	0.82				
RM 1	Rt	<i>Bacillus</i>					0.79
RM 3		<i>Pseudomonas</i>		0.44		0.44	
RM 5		<i>Bacillus</i>		0.42			
RM 7		<i>Chryseobacterium</i>		0.40			
RM 6		<i>Bacillus</i>		0.38		0.44	
TI 26	St	<i>Bacillus</i>	0.72			0.48	0.73
TM 23		<i>Stenotrophomonas</i>		0.46			
TM 30		<i>Bacillus</i>					0.91
TI 21		<i>Bacillus</i>			0.78		
TI 17		<i>Bacillus</i>			0.74		
TM 36		<i>Bacillus</i>				0.46	
FM 5	Fr	<i>Pseudomonas</i>	0.90		0.86		
FI 5		<i>Pseudomonas</i>	0.82				
FI 1		<i>Pseudomonas</i>	0.80				
FM 13		<i>Enterobacter</i>			0.80		
FM 15		<i>Stenotrophomonas</i>			0.80		

Similar strain-dependent and time-dependent inhibitory patterns were observed for *A. alternata* and *F. oxysporum*, confirming a broad antagonistic potential of the selected bacterial isolates against multiple phytopathogens. The temporal dynamics of conidial germination and representative microscopic observations are shown in Figure 4 and Supplementary Figure S2.3.1.

The biocontrol activity of selected bacterial isolates against *Botrytis cinerea* was evaluated in vivo during lettuce seed germination. Seed germination was not affected by either the phytopathogen or the bacterial treatments, with germination rates close to 100% in all experimental groups. In contrast, inoculation with *B. cinerea* alone resulted in a high disease incidence (~80%), with most seedlings exhibiting severe symptoms, including extensive necrosis and high mortality, as reflected by the severity distribution in the bar plot and representative images in Figure 4. The application of bacterial isolates during seed germination significantly reduced both disease incidence and severity in most treatments. With the exception of isolate RhI 22, which did not differ from the pathogen-only control, bacterial inoculation lowered disease incidence to values ranging from 2% to 34%. In these treatments, symptoms were predominantly limited to mild chlorosis, corresponding to the lowest severity category, and seedlings displayed phenotypes comparable to the uninoculated control (Figure 4). The most pronounced biocontrol effects were observed for isolates TM 23 (*Stenotrophomonas* sp.), RM 3 (*Pseudomonas* sp.), and RM 5 (*Bacillus* sp.), which consistently showed the lowest incidence and severity levels, with seedlings maintaining healthy development similar to the disease-negative control, in clear contrast to the extensive tissue decay observed in the pathogen-only and *B. cinerea* + RhI 22 treatments (Figure 4).

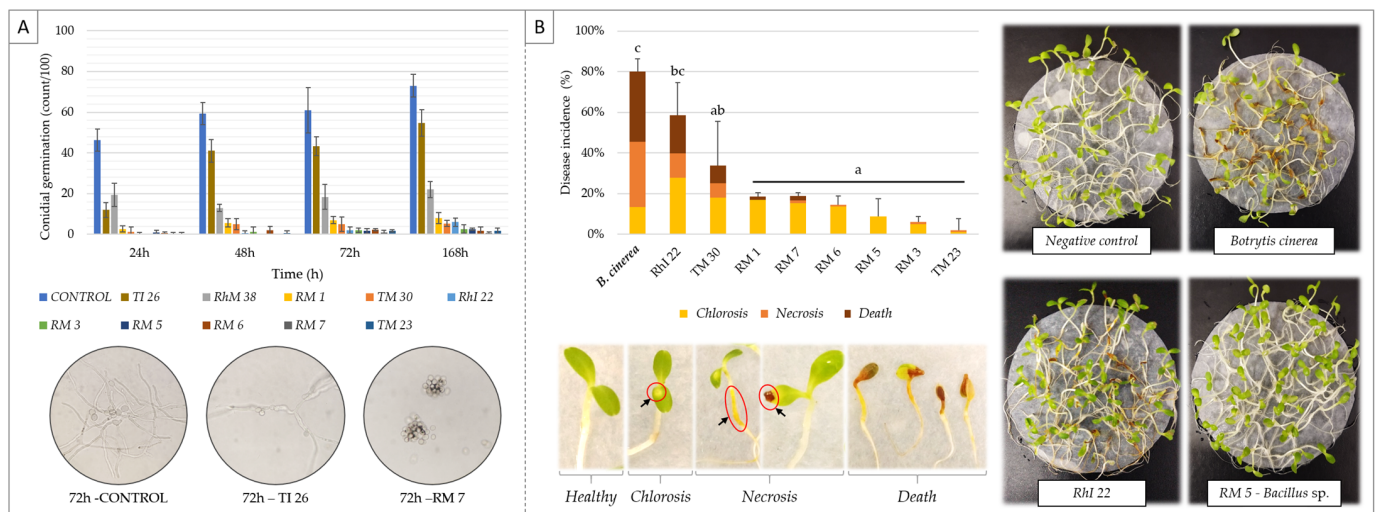


Figure 4. Inhibition of *Botrytis cinerea* conidial germination and biocontrol activity during lettuce seed germination by selected bacterial isolates. **(A)** Counts of conidial germination in the absence (Control) and presence of selected bacterial isolates (TI26, RhM38, RM1, TM30, RhI22, RM3, RM5, RM6, RM7, and TM23) at 24, 48, 72, and 168 h. Bars represent mean values $\pm$ standard error. Representative micrographs illustrate normal germination and hyphal elongation in the control and inhibited germination in the presence of bacterial isolates. **(B)** Disease incidence (%) and severity in lettuce seedlings inoculated with *B. cinerea* and treated with selected bacterial isolates (RhI22, TM30, RM1, RM7, RM6, RM5, RM3, and TM23) during seed germination. Bars represent mean values $\pm$ standard error of the proportion of seedlings exhibiting chlorosis, necrosis, or death. Different letters indicate statistically significant differences among treatments (Tukey test; $p < 0.05$). Representative images illustrating disease severity categories (healthy, chlorosis, necrosis, and death) and overall seedling phenotypes at 5 days post-germination for selected treatments.

3.4. In Vitro and In Vivo Characterization of Plant Growth-Promoting Traits

The characterization of plant growth-promoting traits revealed a broad functional diversity across the bacterial collection, with isolates displaying variable capacities for nutrient mobilization and phytohormone-related activities. The ability to solubilize insoluble phosphate was widely distributed among the tomato-associated bacterial collection. Overall, 42.9% of the isolates exhibited phosphate-solubilizing activity in vitro, indicating a substantial functional potential related to phosphorus mobilization within the collection; these isolates were predominantly recovered from the Rh and tomato endosphere (Figure 5). Considerable variability in phosphate solubilization capacity was observed among positive isolates, with a maximum phosphate solubilization efficiency $PSE = 1.85$; only 11 isolates exceeded PSE values of 1.0, corresponding to halos more than twice the colony diameter. At the taxonomic level, phosphate-solubilizing activity was mainly associated with isolates affiliated with the genera *Pseudomonas*, *Pantoea*, and *Bacillus*.

The screening for siderophore production revealed that this trait was widely distributed across the tomato-associated bacterial isolates. Within the bacterial collection, 101 isolates ($\approx 39\%$ of the collection) exhibited positive siderophore production in vitro, indicating a high prevalence of iron-chelating capacity among the isolates (Figure 5). Siderophore-producing isolates were predominantly recovered from the Rh and from the Rt and St endosphere. Based on the qualitative CAS assay, siderophore-producing isolates displayed variable halo development, ranging from weak to strong production on solid medium. Only three isolates exhibited strong siderophore production, characterized by halos substantially larger than the colony diameter. *Pseudomonas* and *Bacillus* were the taxa most frequently associated with this trait within the collection.

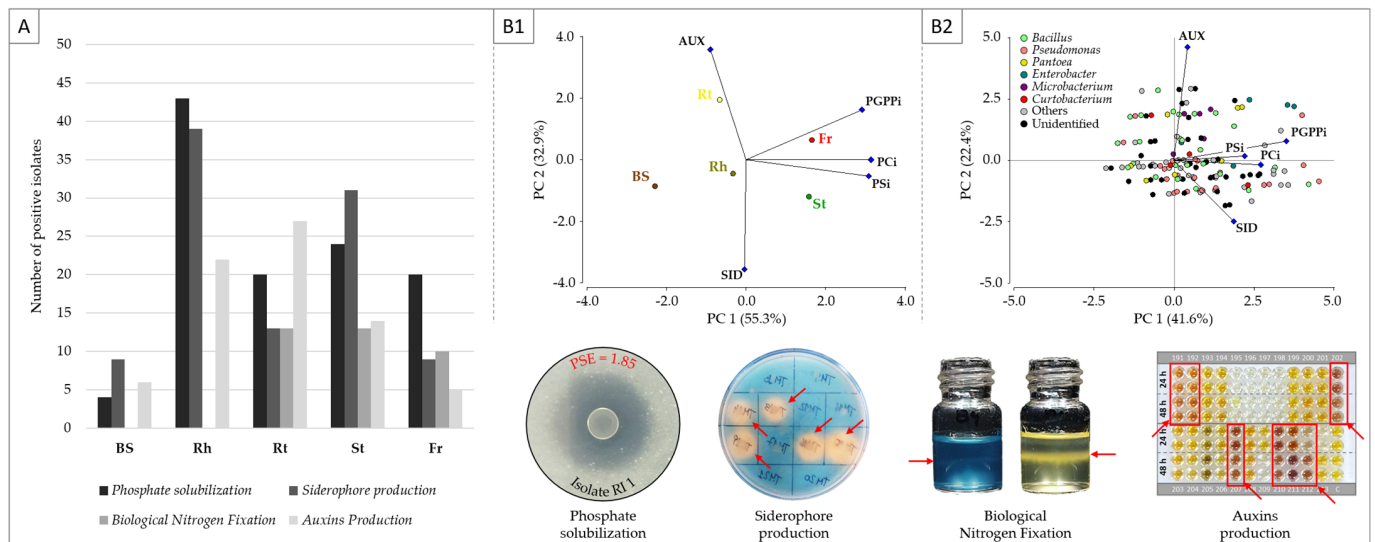


Figure 5. Summary of the in vitro characterization of plant growth-promoting (PGP) traits in the tomato-associated bacterial collection and multivariate analysis. **(A)** Distribution of isolates exhibiting positive responses for phosphate solubilization, siderophore production, biological nitrogen fixation, and auxin production across different microhabitats (BS, bulk soil; Rh, rhizosphere; Rt, root endosphere; St, stem endosphere; Fr, fruit endosphere). Representative examples of in vitro assays are shown, where positive activity is indicated by red arrows. **(B)** Principal component analysis (PCA) performed at the microhabitat (**B1**) and isolate (**B2**) levels using indices related to plant growth-promoting traits and plant colonization: Phosphate Solubilization Index (PSi), Siderophore Production Index (SID), Auxins Production Index (AUX), Plant Colonization Index (PCi), and Plant Growth-Promotion Potential Index (PGPPI).

The in vitro screening of tomato-endosphere-associated isolates for biological nitrogen fixation revealed that this trait was present in a subset of the collection. Overall, 39 out of 160 endophytic isolates (24.4%) exhibited subsurface pellicle formation in nitrogen-free semi-solid media, indicative of nitrogen-fixing capacity (Figure 5). Only two isolates (FM 2 and FM 8), both assigned to *Chryseobacterium*, displayed positive growth in both nitrogen-free media tested (LGI and NFb). Nitrogen-fixing isolates were mainly affiliated with the genera *Bacillus*, *Pseudomonas*, *Chryseobacterium*, and *Pantoea*, and were predominantly recovered from Rt and St endosphere tissues.

The screening for auxin-type phytohormone production revealed that this trait was broadly distributed within the tomato-associated bacterial collection. A total of 74 isolates exhibited a positive response in the colorimetric detection of auxins using Salkowski's reagent, representing approximately 28% of the collection (Figure 5). Auxin-producing isolates displayed variable production levels: 36 isolates showed low production, 23 exhibited moderate production, and 15 were classified as high auxin producers. Auxin-producing isolates, predominantly affiliated with the genera *Bacillus* and *Pseudomonas*, were mainly recovered from the Rh and from the Rt and St endosphere of tomato plants.

Integration of phosphate solubilization, siderophore production, biological nitrogen fixation, and auxin production into the Plant Growth Promotion Traits Index (PGPi) allowed a comparative assessment of the in vitro plant growth-promoting potential across the bacterial collection. Most isolates displayed low index values, with 67 isolates showing a PGPi = 0, indicating the absence of detectable activity in any of the evaluated mechanisms. Only four isolates reached PGPi > 0.5, with the highest score (PGPi = 0.73) recorded for isolate TI 15 (*Pseudomonas*). The 15 isolates with the highest PGPMI values were predominantly recovered from Rt and St endosphere tissues, with fewer representatives from the Rh and Fr endosphere. *Bacillus* and *Pseudomonas* were the most frequently represented

genera among these top-ranked isolates. When PGPi values were integrated with Plant Colonization Index (PCi) through the integrated Plant Growth Promotion Potential Index (PGPPi), isolates exhibiting strong growth-promotion traits also showed favorable colonization capacities.

Principal component analysis (PCA) was used to explore multivariate patterns among plant growth-promotion and colonization-related indices at both the microhabitat and isolate levels (Figure 5). The correlation matrix, together with eigenvalues and the percentage of variance explained by each principal component, is presented in Supplementary Material S2. In the microhabitat-based PCA, the first two components explained 88.2% of the total variance (PC1: 55.3% and PC2: 32.9%). Along PC1, microhabitats were clearly separated, with BS positioned toward negative scores and plant-associated compartments toward positive scores, reflecting higher values of plant colonization capacity and plant growth-promotion indices. PC2 further differentiated microhabitats according to the relative contribution of specific PGP traits, with Rt-associated isolates linked to higher auxin production, whereas St, Rh, and BS compartments were more closely associated with siderophore production. In the isolate-based PCA, the first two principal components explained 64.0% of the total variance (PC1: 41.6% and PC2: 22.4%). PC1 was mainly driven by plant growth-promotion and colonization-related indices, including phosphate solubilization (PSi), siderophore production (SID), plant colonization capacity (PCi), and the integrated plant growth-promotion index (PGPPi), which showed the highest contribution to this axis. Most isolates clustered near the origin, indicating low to moderate index values; however, a subset of isolates clearly separated along the positive side of PC1, reflecting higher overall plant growth-promotion potential. These isolates were predominantly affiliated with the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Pantoea*, and *Curtobacterium*, as well as several taxonomically unassigned strains. PC2 further differentiated isolates according to auxin production (positive scores) and siderophore production (negative scores).

Based on the integrated evaluation of plant growth-promoting traits and colonization capacity, the ten isolates with the highest Plant Growth-Promotion Potential Index (PGPPi) values were selected for subsequent in vivo assays. These isolates were consistently positioned away from the central cluster in the isolate-based PCA (Figure 5), being associated with higher values of plant growth-promotion and colonization-related indices. The selected isolates and their corresponding characteristics are summarized in Table 2.

Table 2. Bacterial isolates selected as plant growth-promotion candidates for in vivo assays based on integrated in vitro indices and multivariate analysis.

Isolate	Microhabitat	Taxonomy	PSi	SID	BNF	AUX	PGPi	PCi	PGPPi
RhM 40	Rh	<i>Bacillus</i>	0.87	+	+	-	0.55	0.25	0.68
RI 17	Rt	<i>Agrobacterium</i>	0.05	+	+	+	0.43	0.50	0.68
RI 13		<i>Peribacillus</i>	0.00	++	+	-	0.42	0.50	0.67
TM 30	St	<i>Bacillus</i>	0.00	++	+	-	0.42	1.00	0.92
TI 15		<i>Pseudomonas</i>	0.91	+	+	++	0.73	0.25	0.85
TM 16		<i>Flavobacterium</i>	0.05	++	+	-	0.43	0.75	0.80
TI 28		<i>Pseudomonas</i>	1.00	+	+	-	0.58	0.38	0.77
TM 31		<i>Enterobacter</i>	0.94	-	-	++	0.40	0.50	0.65
TI 6		<i>Curtobacterium</i>	0.37	++	+	-	0.51	0.25	0.63
FM 13	Fr	<i>Enterobacter</i>	0.21	+	-	+++	0.38	0.50	0.63

PSi: phosphate solubilization index; SID: siderophore production; BNF: biological nitrogen fixation; AUX: auxin production; PGPi: Plant Growth-Promoting Trait Index; PCi: Plant Colonization Index; PGPPi: integrated Plant Growth-Promotion Potential Index. SID and AUX activities were semi-quantitatively categorized as low (+), moderate (++), and high (+++).

Selected candidates were evaluated in vivo for their effects on plant growth in lettuce and tomato at both the seedling and productive stages.

- **Lettuce seedling assay:** In lettuce grown under controlled conditions, selected bacterial isolates significantly affected seedling growth compared with the uninoculated control (Figure 6). Four isolates—TI 15, *Pseudomonas*; TI 28, *Pseudomonas*; RhM 40, *Bacillus*; and TM 30, *Bacillus*—consistently promoted significant increases in total biomass across two independent experiments ($p < 0.05$). Biomass enhancement involved both root and shoot compartments, indicating a generalized stimulation of seedling growth. These isolates also induced a significant expansion of leaf area in the first two true leaves. Among them, TM 30 (*Bacillus*) showed the strongest effect, increasing leaf area by ~210% and total biomass by 50–64% relative to the control. Additional isolates, including RI 13 (*Peribacillus* sp.) and TM 31 (*Enterobacter* sp.), promoted seedling biomass in a single experimental run. Seedling assays conducted under commercial nursery conditions using lettuce cv. Emilia yielded results consistent with those obtained under controlled conditions. Total biomass accumulation before transplanting was significantly higher ($p < 0.05$) in seedlings inoculated with TI 15 (*Pseudomonas* sp.), RhM 40 (*Bacillus* sp.), TM 30 (*Bacillus* sp.), and RI 13 (*Peribacillus* sp.) compared with the uninoculated control (Figure 6). Biomass increases were generally observed in both root and shoot compartments, resulting in improved seedling vigor at transplant.
- **Tomato seedling assay:** In tomato grown under controlled conditions, bacterial isolates resulted in more moderate but significant growth responses (Figure 6). Seedlings inoculated with TM 30 (*Bacillus*) exhibited a significant increase in total dry biomass (+38%) relative to the control ($p < 0.05$), mainly due to enhanced shoot growth. In addition, TI 28 (*Pseudomonas*) and TI 6 (*Curtobacterium*) significantly increased shoot biomass without affecting total biomass. None of the isolates significantly increased root biomass. Among the tested isolates, only TM 30 (*Bacillus*) also promoted a significant increase in leaf area, indicating an early growth-promoting effect at the seedling stage (Figure 6).

In addition, productive-stage assays were conducted under greenhouse conditions at the Estación Experimental Gorina (Ministerio de Desarrollo Agrario, Provincia de Buenos Aires, Argentina). Soil physicochemical properties were suitable for crop development and showed no salinity or sodicity constraints (see Table S2.4.5 in S2. Supplementary data supporting Results).

- **Lettuce productive-stage assay:** Notably, growth-promoting effects observed at the seedling stage were maintained at the productive stage for several bacterial isolates. In both lettuce cultivars tested (cv. Sagess and cv. Emilia), several isolates significantly increased marketable fresh weight compared with the uninoculated control. In cv. Sagess, four isolates enhanced commercial fresh weight by 30–37%: TI 28 (*Pseudomonas*), TM 30 (*Bacillus*), RI 13 (*Peribacillus*), and RhM 40 (*Bacillus*). In cv. Emilia, three isolates promoted increases ranging from 21–32%: TM 30 (*Bacillus*), RhM 40 (*Bacillus*), and TI 15 (*Pseudomonas*) (Figure 7).

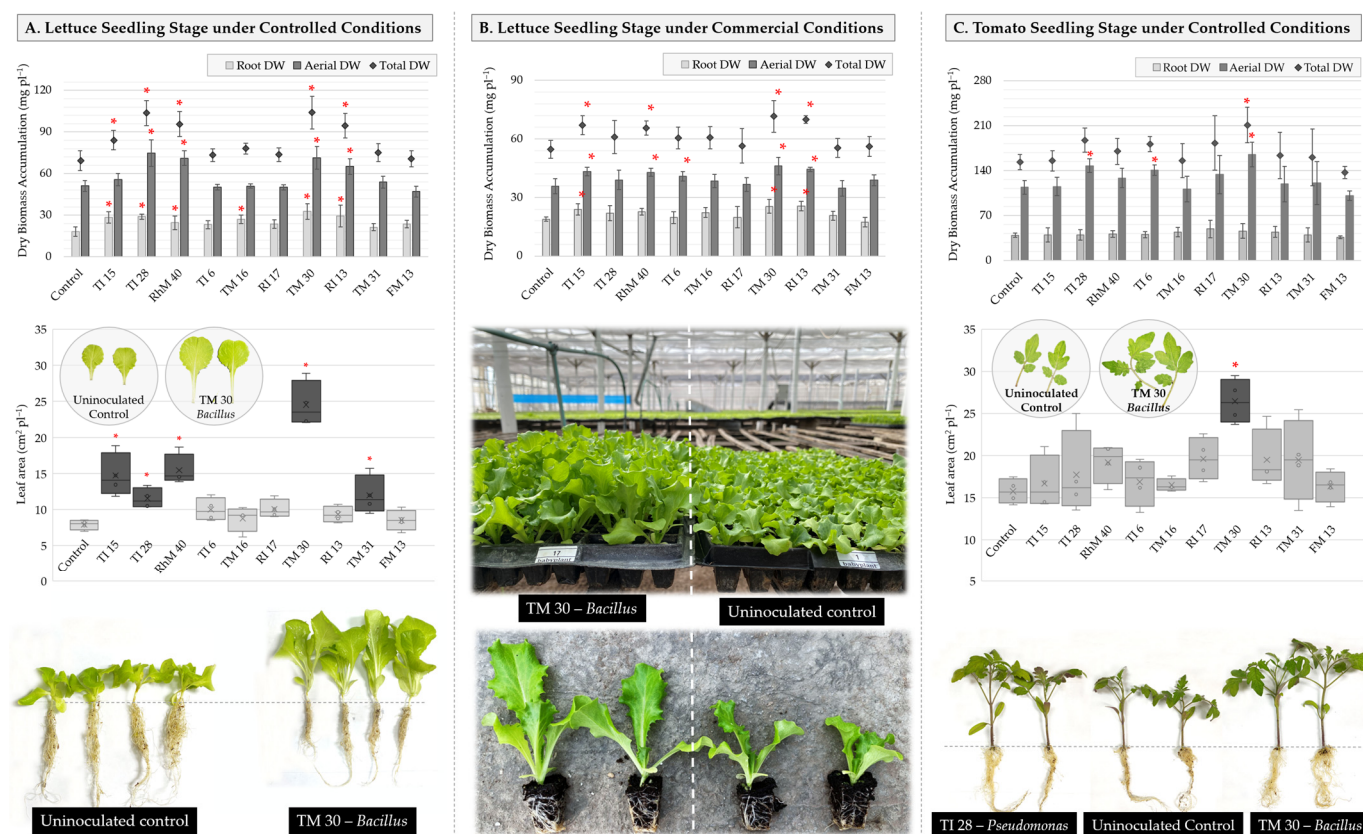


Figure 6. Effects of selected bacterial isolates on seedling growth of lettuce and tomato under controlled and commercial nursery conditions. (A) Lettuce cv. Sagess seedlings grown under controlled conditions; bar plots showing root, shoot, and total dry biomass, and box plots showing leaf area before transplanting. Representative images of bacterial-inoculated and uninoculated plants are shown. (B) Lettuce cv. Emilia seedlings grown under commercial nursery conditions; bar plots showing dry biomass accumulation before transplanting and representative images of inoculated and uninoculated treatments. (C) Tomato cv. Elpida seedlings grown under controlled conditions; bar plots showing root, shoot, and total dry biomass, and box plots showing leaf area before transplanting. Representative images of bacterial-inoculated and uninoculated plants are shown. In all cases, asterisks indicate significant differences relative to the control (Dunnett's post hoc test, $p < 0.05$).

- Tomato productive-stage assay: Cumulative yield was evaluated over six weekly harvests. Across the entire experiment, the mean yield was $3370 \text{ g fruit plant}^{-1}$. Control plants yielded $2971 \pm 170 \text{ g plant}^{-1}$, whereas inoculated treatments ranged from 2991 to $3704 \text{ g plant}^{-1}$ (Figure 7). Although some isolates, including TM 30 (*Bacillus*), RhM 40 (*Bacillus*), and TI 28 (*Pseudomonas*), showed 25.3%, 24.7%, and 21.2% higher yields than uninoculated plants, respectively, bacterial inoculation did not result in statistically significant differences in total yield compared with the control ($p = 0.112$). Fruit number and individual fruit weight were also unaffected by bacterial inoculation. Across treatments, plants produced an average of 32 ± 3 fruits plant^{-1} with a mean fruit weight of $104 \pm 3 \text{ g}$. No significant differences were detected for fruit number ($p = 0.928$) or fruit weight ($p = 0.901$) between inoculated and uninoculated treatments.

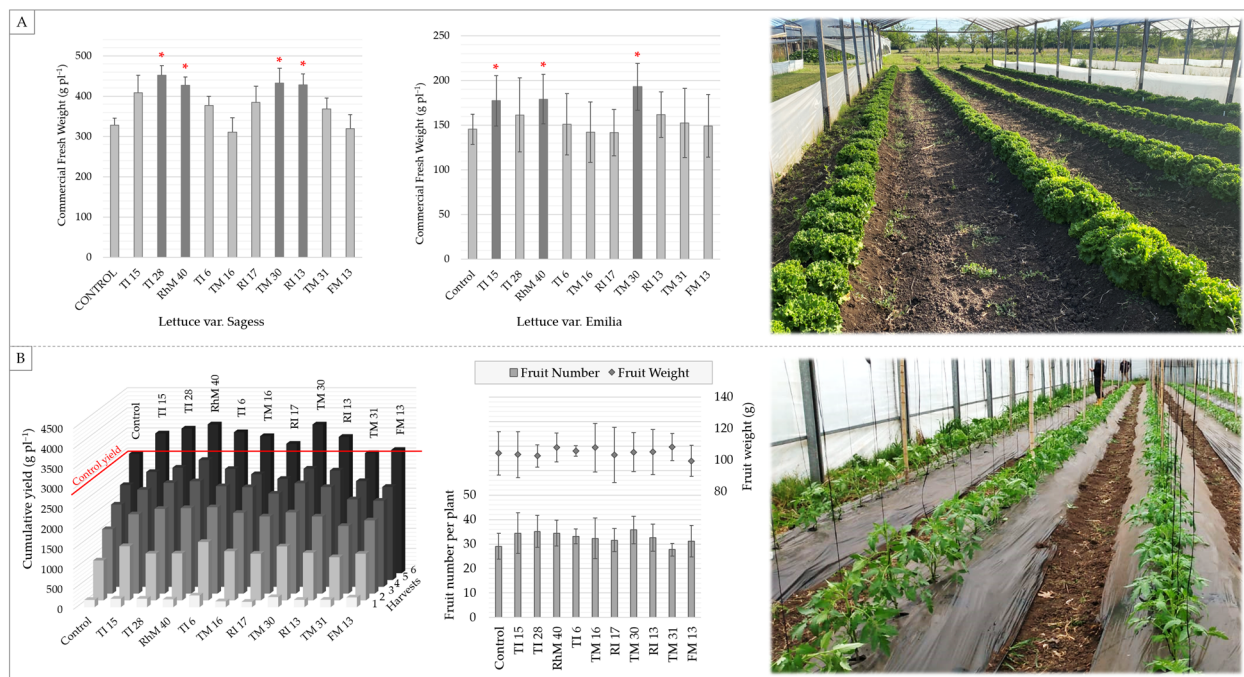


Figure 7. Effects of selected bacterial isolates on lettuce and tomato crop performance during the productive stage under greenhouse conditions. **(A)** Commercial fresh weight of lettuce cv. Sagess and cv. Emilia at harvest following inoculation with selected bacterial isolates. Bars represent mean values $\pm$ SE, and asterisks indicate significant differences relative to the uninoculated control (Dunnett's test, $p < 0.05$). Representative image of lettuce crop at harvest is shown. **(B)** Tomato cv. Elpida productive performance, including cumulative fruit yield over six harvests, fruit number per plant, and mean individual fruit weight. Bars and rhombuses represent mean values $\pm$ SE. Representative image of tomato plants during the cropping cycle is shown.

4. Discussion

4.1. Bioprospecting as a Structured Pathway: From Microbial Diversity to Agronomically Relevant Bioinputs

One of the main challenges in the development of agricultural bioinputs lies not in generating microbial collections per se, but in the rational identification of a reduced subset of isolates capable of delivering consistent and reproducible benefits under realistic field conditions [8,9].

In this context, the present study addresses this challenge, and its contribution extends beyond the identification of promising strains by adopting an approach that differs from traditional selection strategies. Starting from a collection of 259 cultivable bacterial isolates associated with distinct microhabitats of tomato cv. Elpida, the study frames strain selection as a process of rational complexity reduction, demonstrating how large and heterogeneous collections can be systematically transformed into agronomically relevant candidates. The integration of ecological origin, functional characterization, composite indices and multivariate analyses within a structured selection framework enabled the prioritization of isolates based on functional consistency rather than isolated activity. This strategy progressively narrowed the candidate pool, ultimately identifying a small set of isolates that consistently exhibited measurable biocontrol and plant growth-promoting effects in *in vivo* assays.

Within the context of sustainable horticulture and increasing environmental variability associated with climate change, such structured bioprospecting frameworks represent an important step toward bridging the gap between microbial discovery and the development of robust, field-relevant agricultural bioinputs.

4.2. Endophytic Bacteria as a Functional Reservoir: Ecological and Biotechnological Advantages for Bioinput Development

Bacterial abundance was significantly influenced by microhabitat type, with all evaluated microhabitats differing significantly from each other. Along the soil–plant continuum, the rhizosphere (Rh) harbored the highest bacterial loads, significantly exceeding those of bulk soil (BS), reflecting strong selective enrichment at the root–soil interface driven by root exudation and nutrient availability (Figure 1) [34]. As expected, abundance and cultivable diversity decreased toward internal tissues, indicating progressive host-mediated ecological filtering toward the endosphere (Figure 1) [6,34–37].

Despite reduced abundance and morphotype diversity, the endophytic compartments maintained a taxonomically structured assemblage dominated by genera widely associated with plant functions, including *Bacillus*, *Pseudomonas*, *Curtobacterium*, *Microbacterium*, *Chryseobacterium*, and *Pantoea* (Figure 1) [34,38,39]. More importantly, endophytic isolates disproportionately expressed complementary traits related to colonization, antagonism, and growth promotion, including hydrolytic enzyme production, biofilm formation, and multiple PGP mechanisms [40,41]. These features translated into higher composite index values and a greater likelihood of identifying isolates with consistent *in vivo* performance, as also supported by multivariate analyses (Figures 3 and 5). These findings support the interpretation that host-driven selection in the endosphere favors functional compatibility and specialization over sheer diversity, a pattern also documented by microbial community-based and genetic studies reporting decreased diversity and increased specialization toward inner root compartments [42,43]. The endosphere represents a highly selective niche where microbial persistence depends on physiological compatibility and the capacity to provide tangible benefits to the host. Consequently, plant-associated communities may become less diverse but functionally optimized.

Importantly, endophytic origin alone did not guarantee superior performance. While some rhizospheric isolates also exhibited relevant traits, the probability of identifying robust, multifunctional candidates with reproducible *in vivo* effects was higher among endophytes, confirming that ecological origin constitutes a meaningful and non-random criterion in microbial selection strategies [44].

4.3. In Vitro Screening as an Initial Filter Within an Integrative Selection Framework

A key limitation in developing PGPB-based bioinputs is that individual *in vitro* traits often fail to reliably predict plant performance under complex *in vivo* and agronomic conditions, despite their value as initial screening tools [38,44]. This occurs because *in vitro* assays assess microbial functions under simplified and controlled conditions that do not capture the ecological complexity of plant–microbe interactions, including microbial competition, environmental variability, and host-mediated regulation of microbial activity. Moreover, beneficial plant responses are typically the outcome of multifactorial and context-dependent interactions, rather than the expression of single traits in isolation [6,43]. Consistent with previous reports, the tomato-associated bacterial collection generated in this study showed a broad distribution of plant growth-promoting and biocontrol *in vitro* traits (Figures 2, 3 and 5), particularly among genera such as *Bacillus*, *Pseudomonas*, *Enterobacter*, and related taxa [42,45–49].

Importantly, a subset of isolates combining multiple functional traits with strong colonization-related capacities consistently elicited positive responses across independent *in vivo* assays. Representative examples include isolates RM5 (*Bacillus*), TM30 (*Bacillus*), RhM40 (*Bacillus*), and TI15 (*Pseudomonas*), which showed coherent performance across different experimental scales and plant systems (Figures 4, 6 and 7). Notably, these isolates were prioritized based on integrated *in vitro* functional profiles, underscoring the comple-

mentarity between in vitro screening and subsequent in vivo validation in both lettuce and tomato. This complementarity operates across increasing levels of experimental realism: in vitro assays act as mechanistic filters that quantify specific microbial capabilities and reduce the candidate pool; controlled in vivo assays provide early functional validation by confirming translation into measurable plant responses and host compatibility; and evaluations under commercial production conditions serve as an agronomic reality check, testing the stability and practical relevance of observed effects within complex horticultural systems. Collectively, this stepwise framework does not assume direct predictability from single assays but instead builds cumulative evidence to support robust candidate prioritization. Similar integrative approaches have been advocated as more reliable strategies for prioritizing microbial candidates with higher probabilities of success under realistic conditions [42,50,51].

The present study also revealed that several isolates exhibiting positive in vitro activities failed to produce measurable effects on disease suppression or plant growth when evaluated in planta. This reinforces the view that in vitro assays should be regarded as necessary but not sufficient filters, rather than as standalone predictors of agronomic performance.

4.4. Consistency and Limits of PGPB Effects Across Horticultural Growth Stages

One of the most critical aspects in the evaluation of plant growth-promoting bacterial candidates is the consistency and durability of their effects across developmental stages and production scales. While numerous studies report positive responses at early growth stages under controlled conditions, far fewer extend validation to productive systems, where plant physiology, resource allocation, and environmental variability substantially increase system complexity [29,38,52].

In the present study, a consistent growth-promoting pattern was observed during the seedling stage in both lettuce and tomato (Figure 6). Several isolates selected through the integrative framework significantly enhanced biomass accumulation and leaf area, and these responses were confirmed under commercial nursery conditions for lettuce, reinforcing their agronomic relevance. Early seedling vigor is a key determinant of transplant establishment, stress resilience in intensive horticulture [53].

Under productive conditions, responses differed between crops (Figure 7). Lettuce exhibited clear and reproducible increases in marketable fresh weight (21–37%) across cultivars, particularly with isolates TM30 (*Bacillus*), RhM40 (*Bacillus*), and TI15 (*Pseudomonas*). In contrast, tomato showed positive but non-significant yield trends. Rather than indicating failure, this difference likely reflects the higher physiological and agronomic complexity of fruit crops, where yield integrates multiple interacting factors and may buffer microbial effects [44,54,55].

From a methodological perspective, the absence of significant yield increases in productive tomato assays highlights the importance of incorporating realistic agronomic expectations into PGPB evaluation pipelines. Yield is an integrative and highly variable trait, often less sensitive to microbial inoculation than early growth parameters, particularly under well-managed greenhouse conditions. Leafy vegetables such as lettuce are more sensitive to improvements in nutrient uptake and vegetative growth, whereas fruit crops like tomato integrate multiple physiological and environmental processes into yield, which can buffer microbial effects on final production [38,54,55]. Moreover, tomato plants were inoculated only at sowing and transplanting and harvested approximately five months later, a timeframe that may dilute early microbial effects, highlighting the need to explore inoculation strategies that reinforce and maintain plant-bacteria interactions over the crop cycle. Similar stage-dependent attenuation of PGPB responses has been reported elsewhere [42,50] and has been attributed to reduced niche availability as native microbial

communities establish, lower persistence of inoculated strains under variable field conditions, and the context-dependent nature of hormone- and nutrient-mediated growth responses.

Crucially, despite differences in the magnitude of responses between stages, the same bacterial isolates consistently ranked among the top candidates across seedling and productive assays. This convergence supports the robustness of the selection framework, as it did not generate stage-specific false positives but instead identified isolates with stable performance tendencies across increasing levels of biological complexity. Overall, these results highlight the value of multi-stage *in vivo* validation for capturing both the consistency and the limits of PGPB effects under realistic horticultural conditions. Together, these findings further demonstrate the complementarity between the *in vitro* screening and the *in vivo* lettuce and tomato assays, supporting the internal coherence of the proposed selection framework.

4.5. *Bacillus* and *Pseudomonas* as Outcomes of Performance-Based Selection

Throughout the bioprospecting pipeline, only a subset of strains from multiple bacterial genera recovered from plant tissues met the combined criteria of functional trait expression and consistent *in vivo* performance. Under these constraints, *Bacillus* and *Pseudomonas*—genera with well-documented biocontrol and plant growth-promoting capacities—isolates consistently ranked among the highest-performing candidates, whereas other taxa failed to sustain performance across multiple evaluation stages (Figures 4, 6 and 7). This result supports the view that the predominance of these genera reflects functional robustness under diverse experimental and agronomic conditions rather than conventional taxonomic bias [38,56].

Notably, although *Bacillus* and *Pseudomonas* accounted for the majority of isolates showing consistent *in vivo* activity, the integrative selection framework also revealed a smaller set of effective strains belonging to other genera, including *Chryseobacterium*, *Stenotrophomonas*, *Curtobacterium*, *Flavobacterium*, and *Enterobacter*. While comparatively less represented among top-ranked candidates, these taxa produced statistically significant biocontrol or plant growth-promoting effects in at least one *in vivo* assay. This finding indicates that, despite the convergence of functional robustness within a limited number of well-adapted genera, the tomato-associated microbiome retains phylogenetically diverse lineages with exploitable agronomic potential. This broader detection capacity represents an important advantage of the integrative selection framework, as it expands the diversity of candidate microorganisms, increases the probability of identifying complementary functional traits and may contribute to the development of more robust and environmentally adaptable microbial bioinputs.

4.6. Bridging Microbial Potential and Agronomic Performance Through Structured Selection

The high selection accuracy obtained demonstrates the effectiveness of the approach employed in this study. From an initial collection of 259 isolates, the integrative pipeline—based on composite indices, multivariate analyses, and ecological context—led to the selection of 18 candidates for *in vivo* evaluation (8 for biocontrol and 10 for plant growth promotion). Notably, 15 of these isolates produced statistically significant effects relative to the uninoculated control in at least one *in vivo* assay, either in disease suppression or plant growth promotion. This success rate highlights the capacity of the framework to objectively prioritize biologically relevant candidates, a key requirement for efficient bioinput development.

The use of integrated indices, such as PBi and PGPPi, further strengthens this approach by consolidating multiple functional and phenotypic traits into quantitative descriptors of

strain performance. This facilitates objective ranking and comparison among candidates, improving the translational value of microbial bioinput research [43,57], an aspect still rarely implemented in applied horticultural studies.

Importantly, these composite indices were conceived as standardized and uniformly applied comparative tools—rather than universal predictive models—designed exclusively to support objective internal prioritization of isolates based on experimentally derived traits. Although validated using tomato and lettuce as model systems, the framework is not crop-specific. Its focus on functional relevance, ecological competence, and early-stage performance consistency supports its transferability to other intensively managed horticultural crops, providing a scalable and reproducible model for performance-based PGPB selection.

4.7. Limitations and Future Perspectives

Despite the robustness of the proposed framework and the consistency of the results obtained across multiple evaluation stages, several limitations should be acknowledged. Recognizing these constraints is essential not only for a balanced interpretation of the findings but also for guiding future research and development toward deployable bioinputs.

First, this study focused exclusively on the cultivable fraction of the tomato-associated microbiota. While culture-dependent approaches remain indispensable for the development of microbial bioinputs, inherently select for microorganisms capable of growing under the imposed laboratory conditions (e.g., culture medium composition, incubation temperature, and duration) and therefore represent a methodologically filtered subset of the natural microbial community [58]. Consequently, a substantial proportion of microbial diversity may remain undetected, potentially contributing to plant performance through indirect or community-level interactions [59]. The integration of culture-independent tools, such as amplicon sequencing or functional metagenomics, could provide complementary insights into microbial community structure and help refine future isolation and selection strategies.

Second, *in vivo* validation was conducted under a limited number of environmental and production conditions, including a single greenhouse system. Although this design was sufficient to test the internal consistency of the framework and to identify robust early-stage candidates, microbial performance is known to be highly context-dependent, influenced by soil properties, crop management, climate, and host genotype [38,43]. Moreover, a common inoculation scheme was applied across crops with markedly different production cycles, ranging from short-cycle lettuce (~70 days) to long-cycle tomato (~5–6 months). While this standardized approach enabled comparative evaluation, it may also have attenuated microbial effects in longer production systems, underscoring the need for crop- and stage-specific inoculation strategies in future studies.

Third, the present work did not track the persistence, population dynamics, or spatial distribution of the inoculated strains within plant tissues or the rhizosphere. As a result, the precise relationship between colonization patterns and observed phenotypic effects remains unresolved. Similarly, although functional traits were characterized *in vitro*, the specific mechanisms responsible for the *in vivo* growth-promoting or biocontrol effects of individual isolates were not determined. Targeted molecular, physiological, and microbiome-based analyses will be required to resolve these mechanisms and to link functional expression to ecological performance.

Fourth, formulation aspects were not optimized for individual isolates. Formulation, carrier selection, and shelf-life are known to strongly influence microbial survival and field performance [60], and their omission likely limited the magnitude and persistence of the observed effects. Importantly, the collection generated here provides a valuable resource for

the rational development of multi-species bioinputs, which often outperform single-strain inoculants due to functional complementarity and increased ecological stability [29].

Finally, biosafety considerations were beyond the scope of this study. The pathogenic potential of the selected isolates, particularly with respect to human health, as well as their impact on the native plant microbiome, was not evaluated. These aspects are essential prerequisites for regulatory approval and commercial deployment and should be explicitly addressed in subsequent development stages.

Despite these limitations, the present work provides a strong proof of concept that a structured, performance-driven bioprospecting framework can effectively identify microbial candidates with reproducible *in vivo* activity. Future efforts that integrate formulation science, ecological monitoring, and biosafety assessment will allow these candidates to move from experimental validation toward practical, sustainable microbial bioinput development.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16050610/s1>, S1: Supplementary material supporting Materials and Methods; S2: Supplementary material supporting Results; S3: Supplementary material of Bacterial Isolated Collection.

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Abbreviations

The following abbreviations are used in this manuscript:

Aa	<i>Alternaria alternata</i>
APi	Antagonistic Potential Index
AUX	Auxins Production Index
Bc	<i>Botrytis cinerea</i>
Bf	Biofilm Formation Index
BNF	Biological Nitrogen Fixation Index
BPi	Biocontrol Potential Index
BS	Bulk Soil
CFU	Colony-forming Units
CHP	Horticultural Belt of La Plata

ECi	Enzymatic Characterization Index for Plant Colonization
ECiA	Enzymatic Characterization Index for Antagonism
Fo	<i>Fusarium oxysporum</i>
Fr	Fruit Endosphere Samples
PCi	Plant Colonization Index
PGPB	Plant Growth-Promoting Bacteria
PGPi	Plant Growth-Promoting Trait Index
PGPPi	Plant Growth-Promotion Potential Index
PIRG	Percentage of Inhibition of Radial Growth
PSi	Phosphate Solubilization Index
Rh	Rhizosphere Samples
Rt	Root Endosphere Samples
SID	Siderophore Production Index
Ss	<i>Sclerotinia sclerotiorum</i>
St	Stem Endosphere Samples

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