

Selected putative endophytic bacteria from sweet pepper: Antagonistic activity against *Escherichia coli* and *Listeria innocua*, and their plant growth promoting traits

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ABSTRACT

The increasing demand for raw and minimally processed agricultural products has highlighted the necessity for natural strategies that enhance crop performance and reduce microbial risks along the food production chain. The objective of this study was to select and characterise bacterial strains having biocontrol and plant growth-promoting (PGP) traits from putative endophytes of surface sterilised sweet pepper (*Capsicum annuum* L var. *grossum*). Amongst the 42 bacterial strains examined, seven exhibited antagonistic activity against either *Escherichia coli* ATTC 8739 or *Listeria innocua* CCM 4030^T, with two of these bacteria inhibiting both surrogate food-borne pathogens. The inhibitory strains were identified by

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sequencing the 16S rRNA and *rpoB* genes, revealing five *Pseudomonas*, one *Enterobacter* and one *Bacillus* species. In tests of PGP traits, such as the indole-3-acetic acid (IAA) production, phosphate and zinc solubilisation, and cellulase activity, *Pseudomonas protegens* SESK2 exhibited the strongest potential. These findings demonstrate the dual functionality of the selected putative endophytic bacteria, and highlight their potential application in sustainable agri-food systems.

KEYWORDS

biocontrol, putative endophytic bacteria, food safety, plant growth promotion, sweet pepper

1. INTRODUCTION

The incidence of foodborne outbreaks linked to raw plant-based produce has been steadily increasing. According to the most recent EU One Health Zoonoses Report (EFSA and ECDC, 2024), infections caused by Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* represented the third and fifth most reported zoonotic diseases, respectively. This escalation can be attributed, at least in part, to the increasing distribution and consumption of fresh, minimally processed, and ready-to-eat produces, which provide potential reservoirs for a wide range of pathogens (Belák et al., 2014). While the prevalence of *L. monocytogenes* in fresh fruits and vegetables is lower compared to foods of animal origin and certain other ready-to-eat (RTE) foods (EFSA BIOHAZ Panel, 2018; Lepecka et al., 2022), pre-packaged salads and fresh-cut leafy vegetables can support the growth of it during refrigerated storage (Culliney and Schmalenberger, 2020; Abdul et al., 2026); therefore, these products receive special attention in EU risk assessments. *E. coli* is more commonly found in horticultural products, particularly in leafy vegetables. Although the prevalence of pathogenic STEC/EHEC (Shiga toxic or enterohaemorrhagic *E. coli*) strains is generally low, contamination of sprouted seeds with pathogenic *E. coli* is a major source of foodborne diseases (Barak et al., 2002). Nevertheless, leafy vegetables, pre-packaged salads and sprouted seeds pose a significant food safety risk, as they can provide favourable conditions for the survival and proliferation of certain enteric pathogens (EFSA, 2025).

Pathogenic microorganisms originating from the gastrointestinal tracts of human and animals are widely disseminated into the environment via faecal material and contaminated water, eventually re-entering the food chain and contributing to human diseases. In addition, pathogenic bacteria can contaminate plants through untreated irrigation water, insect vectors, or human handling during harvesting. Once introduced, these pathogens may associate with plants and adapt to persist for extended periods (Govindarajan et al., 2023). Furthermore, they can enter the internal plant tissues, where they interact with the resident microbial communities (Teplitski et al., 2011).

The plant microbiota encompasses a wide range of microbial taxa, including beneficial, commensal, and pathogenic species. Within these communities, certain bacteria play particularly important roles by enhancing plant growth, providing protection under adverse environmental conditions, alleviating stress, and facilitating nutrient acquisition, which are commonly designated as plant growth-promoting (PGP) bacteria (Chialva et al., 2022). Two major

categories of plant-associated bacteria have been identified as possessing PGP potential: those colonising the rhizosphere and those occurring as endophytes (Santoyo et al., 2016).

Endophytic bacteria inhabit the internal plant tissues without eliciting harmful effects. Their direct roles in promoting plant growth include nutrient acquisition, modulation of phytohormone levels (e.g., indole-3-acetic acid; IAA), and enhancement of the availability of limiting elements such as phosphorus and zinc. Phosphorus, although essential for plant development, often exhibits limited bioavailability due to its chemical nature. In soils, the majority of phosphorus occurs as phosphate, typically in the form of metal complexes bound to minerals or organic materials. Consequently, the microbial solubilisation of inorganic phosphate represents a critical mechanism by which plant-associated microorganisms can support plant growth (Varga et al., 2020). In many soils, the fraction of zinc available for plant uptake is very limited, as the bioavailable ionic form presents only trace amounts. Zinc solubilisation by plant-associated bacteria can occur through multiple mechanisms, including the secretion of organic acids, proton extrusion and the production of chelating compounds (Eshaghi et al., 2019).

Endophytic bacteria can also indirectly enhance host plant growth and fitness by producing antibiotics and cell wall degrading enzymes, as well as by activating plant defence responses against phytopathogens. Researchers have demonstrated that a wide range of endophytic bacteria possesses the capacity to impede the proliferation of foodborne pathogenic bacteria (Das et al., 2017; Islam et al., 2018). Numerous studies provide evidence for the inhibitory effect of endophytes against pathogenic bacteria, such as the *E. coli* O157:H7 and *L. monocytogenes*. This effect is primarily due to the penetration of secondary metabolites through existing or newly formed pores in the cell membrane, resulting in altered metabolic functions, the efflux of intracellular substances from the cell, cell lysis, and cell death (Kotzekidou et al., 2008; Das et al., 2017; Islam et al., 2018, 2019). Among the inhibitory metabolites produced by the endophytic bacteria, various antibiotics have proven to be of particular interest. Polymyxin, a cationic antibacterial peptide, has been demonstrated to be capable of degrading the outer membrane of Gram-negative bacteria (Wang et al., 2022). Moreover, its inhibitory effect has been demonstrated not only against *E. coli* O157:H7, but also against *L. monocytogenes* (Shehata et al., 2017).

The PGP effect is exerted by the endophytes on a range of plants, since they are not host-specific. This makes them promising candidates for use as biofertilisers (Chaudhary et al., 2022).

Several strategies target the suppression of growth and the limitation of the dissemination of human pathogenic bacteria in cultivated plants, as well as in fresh fruits and vegetables. Recognition of the antagonistic interactions between certain endophytes and food-borne pathogens has opened the possibility of exploiting endophytic bacteria as biocontrol agents. While the antagonistic effects of endophytic bacteria against human pathogens have been widely demonstrated, the detailed mechanisms underlying these interactions require further research activity (Akhtyamova, 2013; Wang et al., 2024). Expanding this knowledge base is expected to facilitate the development of more targeted biocontrol products, ultimately contributing to more effective protection of raw and processed food commodities from pathogenic contamination.

In many laboratory studies involving pathogenic bacteria, the pathogens themselves are not used directly; instead, surrogate bacteria are employed to assess the relevant effects. A surrogate is defined as a bacterium that replaces the target pathogen in experimental settings. As reviewed by Sinclair and colleagues (Sinclair et al., 2012), both pathogenic and non-pathogenic microorganisms can serve as surrogates for various purposes, including studies on growth and survival under specific food processing and distribution conditions, evaluation of disinfection, method

development, and as indicators of particular environment. Although the direct use of pathogens can yield the most representative and reliable results, this approach is often limited by biosafety concerns. The use of surrogates mitigates many of the human and environmental safety risks associated with handling pathogens in association with plant cultivation and processing (Park et al., 2018).

The aim of this study was to identify and characterise a distinct population of endophytic bacteria isolated from sweet pepper (*Capsicum annuum* var. *grossum*) and selecting strains with a potential of inhibiting the growth of foodborne pathogenic bacteria and exhibit *in vitro* plant growth-promoting activity (PGPA), thereby representing potential candidates as effective biocontrol agents. Putative endophytes obtained from surface-disinfected tissues of sweet pepper plants were tested and evaluated for their inhibitory effects against non-pathogenic *E. coli* and *Listeria innocua*, which served as surrogates for pathogenic *E. coli* (e.g., *E. coli* O157:H7) and *L. monocytogenes*, respectively. In addition, unique PGP traits of the antagonistic strains – including cellulose degradation, phosphate and zinc solubilisation and indole-3-acetic acid production – were also investigated.

2. MATERIALS AND METHODS

2.1. Isolation of endophytic bacteria from sweet pepper

The isolation of endophytic bacteria from the 'Kárpia' and 'Hó' cultivars of sweet (bell) pepper (*Capsicum annuum* L var. *grossum*) was conducted in both soil and hydroponic cultivation settings. 'Kárpia' and 'Hó' are registered cultivars in Hungary and the EU under the IDs 186049 and 300722, respectively (National Food Chain Safety Office, 2012). The plants were cultivated at the Experimental and Research Farm of the Hungarian University of Agriculture and Life Sciences, Budapest, Hungary. The isolation experiments spanned from 2012 to 2014.

Isolation of endophytic bacteria from pepper plants was conducted following the widely used surface disinfection with ethanol and sodium hypochlorite (Hartmann and Baldani, 2006), as described by Belák et al. (2014). Sterility of the samples was verified by plating out aliquots from the last rinsing water on nutrient agar (Biokar Diagnostics, Budapest, Hungary), in order to ensure that the surface of the treated plant organs was free from living microorganisms. Disinfected parts of the plant organs were incubated in 5 mL sterile PGYS (peptone 5 g L⁻¹, glucose 1 g L⁻¹, yeast extract 2.5 g L⁻¹ and sodium chloride 5 g L⁻¹) broth supplemented with Nystatin (0.1 g L⁻¹) at 25 ± 1 °C by a test tube rotator for 7 days at 25 °C under aerobic conditions. A loopful broth from each test tube exhibiting visible growth was streaked on PGYS agar plate. Following a three-day long incubation at 25 °C, colonies exhibiting varying morphologies were isolated. Individual colonies were inoculated to PGYS agar slants and stored at 4 °C until the commencement of the assays. Long-term maintenance of the isolates was conducted in PGYS broth containing 20% glycerol at -80 °C.

Phenotypic characterisation and RAPD-PCR genotyping of the isolates, followed by cluster analysis, were performed as described in Belák et al. (2014). In the frame of the OTKA K-101716 project (National Research, Development and Innovation Office, NKFI) a total of 288 putative endophytic bacteria were isolated and assigned to 200 clusters according to their phenotypic and genotypic profiles. Based on this clustering, 42 representative isolates were selected for the

analyses presented in this study. The results of the comprehensive analysis of the putative endophytic bacterial populations of sweet pepper are under publication.

2.2. Determination of the antagonistic effect of endophytic isolates against surrogate food-borne pathogenic bacteria

For inhibition activity of the non-pathogenic *E. coli* ATTC 8739 (American Type Culture Collection, Gaithersburg, Maryland, USA) and *L. innocua* CCM 4030^T (Czech Collection of Microorganisms, Brno, Czech Republic) were tested as surrogates of pathogenic *E. coli* strains and *L. monocytogenes*, respectively.

Overnight cultures of *E. coli* and *L. innocua* were prepared in Tryptone Soy Broth (TSB, Biokar Diagnostics, Budapest, Hungary) at 37 °C and used for making cell suspensions of OD 0.5 at 600 nm, corresponding to approximately 10^8 cells mL⁻¹. Four-member decimal dilution series were made from the stock solutions and 1 mL from the last members of the dilutions were inoculated onto the surface of STSA plates (Tryptone Soy Agar [TSA; Biokar Diagnostics, Budapest, Hungary] supplemented with 5 g L⁻¹ NaCl). After spreading the cell suspensions evenly, the excess was removed with a pipette, and the plates were let to dry.

Endophytic strains were cultured in PGYS broth overnight at 25 °C, then suspensions were prepared in sterile distilled water to get OD_{600 nm} = 0.5 (ca. 10^8 cells mL⁻¹). Ten µL aliquots from 100-times diluted suspensions were pipetted onto STSA plates in spot, which were inoculated formerly with either *E. coli* or *L. innocua*. Plates were incubated at four different temperatures such as 20, 25, 30 and 37 °C. Growth of the inoculated bacteria and formation of inhibition zones were recorded every 24 h for 3 days. The experiments were carried out in three replicates.

2.3. Molecular identification of the inhibitory endophytic bacteria

Genomic DNA of the endophytic strains was extracted from PGYS broth overnight cultures using the MasterPureTM Complete DNA and RNA Purification Kit (Epicentre, Madison, Wisconsin, USA).

PCR amplification and sequencing of the 16S rRNA and the *rpoB* genes was performed as described in Belák et al. (2014). Nucleotide sequences of the 16S rDNA amplicons and that of the 16S rRNA gene deposited in the NCBI (National Center for Biotechnology Information) Nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>) were aligned by the BLAST program and algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Camacho et al., 2009) and the similarity ratio was calculated. Amplified 16S rDNA sequences of the endophytic strains were also aligned with that of the type strains deposited in the EzBioCloud database and identified (<https://www.ezbiocloud.net/identify>) (Yoon et al., 2017). The *rpoB* gene was analysed only in those strains, which were found to belong to the *Pseudomonas* genus. In this case, nucleotide sequences of the *rpoB* gene deposited in the NCBI Nucleotide database were used for the alignment with the *rpoB* sequenograms of the strains to be identified. Sequences of 16S rRNA and *rpoB* genes of the endophytic strains were deposited in the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

2.4. Depository and availability of the investigated strains

The bacterial strains used in this study are deposited in the collection of the corresponding author's department and are available upon reasonable request under specific material transfer agreements.

2.5. In vitro screening of the endophytic bacterial strains for plant growth-promoting activities (PGPA)

For all PGPA experiments, the endophytic strains were cultured in PGYS broth overnight at 25 °C. Suspensions were then prepared in sterile distilled water to achieve an OD₆₀₀ of 0.5 (approximately 10⁸ cells mL⁻¹). These suspensions were used to inoculate the agar plates. Three parallel measurements were conducted for each test.

2.5.1. Detection of indole-3-acetic acid (IAA) production. JNFb medium (Shrivastava and Kumar, 2011) was used for screening of the IAA producing endophytic bacteria. The inoculated agar plates were incubated for 7 days at 25 °C. After this period the plates were layered with 50:1 mixture of perchloric acid and iron (III) chloride. The change in colour to pink indicated the IAA production.

2.5.2. Phosphate solubilisation assay. Phosphate solubilisation ability of the strains was tested on Pikovskaya's agar (Sigma Aldrich, Merck, St. Louis, USA), which contained insoluble phosphate. The inoculated plates were incubated for 7–10 days at 25 °C. A positive result was indicated by the development of a clear zone or halo around the macrocolonies.

2.5.3. Zinc solubilisation assay. Zinc solubilisation was tested using a modified culture medium as described by Hartmann and Baldani (2006). Cell cultures of the tested endophytic strains were inoculated and the plates were incubated for 5 days at 25 °C. Strains that established distinct clearing zones around the macrocolonies were considered zinc-solubilising.

2.5.4. Detection of cellulolytic activity. Cellulolytic ability of the endophytes was tested on Congo-red agar medium (Sazci et al., 1986). The inoculated agar plates were incubated for 5 days at 25 °C. The presence of a clear zone or halo around the macrocolonies indicated positive cellulolytic activity.

2.6. Statistical analyses

In addition to descriptive statistics, one- and two-factor repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test was used to compare the inhibition effect of the strains and temperature levels. All statistical analyses were performed using IBM SPSS Statistics 29.0.1.0.

3. RESULTS AND DISCUSSION

3.1. Antagonistic effect of putative endophytic bacterial strains against *E. coli* and *L. innocua*

L. innocua CCM 4030^T and *E. coli* ATCC 8739 are regarded as suitable surrogate models for *L. monocytogenes* and pathogenic *E. coli* (e.g. EHEC strains), respectively, due to their high degree of genetic similarity, comparable growth dynamics, and – given that they can be found in similar environments – analogous resistance to environmental stresses such as heat, acid, and sanitisers, without exhibiting equivalent safety concerns (Mohan et al., 2019;

Gradovska et al., 2022; Gėdas et al., 2024). Consequently, the results that have been obtained by employing surrogate bacteria can be adapted to the obligate pathogenic bacterial species.

Altogether 42 putative endophytic bacterial strains originated from different tissues of hydroponic and soil cultures of ‘Hó’ and ‘Kárpia’ cultivars of sweet pepper were included in this study. Of the 42 putative endophytes tested, 14 (33.3%) created visible inhibition zones against *E. coli* ATCC 8739 under the screening conditions. However, only four strains (SEFPRK4, SEPRK29, SEFPRK2 and SESK2, accounting for 9.5% of the total) consistently formed clear inhibition zones across all three replicates. Against *L. innocua* CCM 4030^T, 19 strains (45.2%) demonstrated observable inhibitory effects, yet only five isolates (11.9%) produced well-defined inhibition zones (SEPRK15, SEPRK19, SEFLH1, SEFPRK2 and SESK2) (see Table 1 and Supplementary Material). Among all isolates tested, only two strains – SEFPRK2 and SESK2 (representing 4.8 %) – consistently effected complete inhibition of both surrogate bacteria.

These results underscore that while many endophytic strains can influence the growth of free-living bacteria, only a subset is capable of robust and reproducible inhibition. This narrow “effective subset” is consistent with findings in other endophyte screening studies, where only a minority of isolates displayed strong inhibitory phenotypes under *in vitro* conditions. For instance, Chantapakul et al. (2024) observed 16.8% inhibition against *L. monocytogenes* among endophytes from tropical fruits, a rate that is broadly comparable to our 11.9% for *L. innocua*.

When incubation temperature was included as a variable, five treatment–strain combinations exhibited clear inhibition zones for *E. coli*. One strain (SEFPRK4) exhibited inhibition at two temperatures: 25 and 30 °C. Of these, we included the data from the 25 °C measurement, which exhibited greater inhibition in the analysis. Two combinations (SEFPRK2 at 25 °C and SESK2 at 20 °C) resulted in inhibition on the 1st, 2nd and 3rd days; the others showed inhibition only on the 2nd and 3rd days. For this reason, only the data from the 2nd and 3rd days were included in the repeated-measures ANOVA. It confirmed strong effect of treatments

Table 1. Characteristics of the antagonistic putative endophytic bacterial (PEB) strains exhibiting complete inhibition of *E. coli* ATTC 8739 and *L. innocua* CCM 4030^T

Inhibited strains	PEB strains		Inhibition		Biggest clearing zone formation		
	Number	ID	Temperature range (°C)	Minimum day(s)	Diameter (mm)	Time (days)	Temperature (°C)
<i>E. coli</i> ATTC 8739	5.	SEFPRK ^a 4	25, 30	2	8.0	3	25
	15.	SEPRK ^b 29	20	2	2.5	2, 3	20
	31.	SEFPRK ^a 2	25	2	5.5	2, 3	25
	35.	SESK ^c 2	20	1	4.5	2	20
<i>L. innocua</i> CCM 4030 ^T	2.	SEPRK ^b 15	20, 25, 30	2	3.5	2, 3	20, 25
	3.	SEPRK ^b 19	20, 25, 30	2	1.5	2, 3	20, 25
	11.	SEFLH ^d 1	20, 25, 30, 37	1	4.0	2	20
	31.	SEFPRK ^a 2	20, 25	1	7.0	2	25
	35.	SESK ^c 2	20, 25, 30, 37	1	12.0	1, 2, 3	20, 25

^a: main root of soil-grown Kárpia cultivar.

^b: main root of hydroponic Kárpia cultivar.

^c: steam of hydroponic Kárpia cultivar.

^d: leaf of soil-grown Hó cultivar.

($F(3,8) = 275.8, P < 0.001$), and post hoc tests revealed that all four strains (when tested at their respective temperatures) differed significantly from the others ($P < 0.001$; Fig. 1). The temperature dependence suggests that the antagonistic activity is highly sensitive to environmental conditions. Some strains may only produce antagonistic compounds or maintain their inhibitory potential within a narrow thermal range (Wang et al., 2024).

In the case of *L. innocua*, a two-factor repeated-measures ANOVA revealed a strong interaction ($F(8,30) = 174.7, P < 0.001$) between the strains and temperatures. Both the incubation time–strain interaction and the main effect of incubation time (Day 2 vs Day 3) were significant, while the incubation time–temperature interaction was not. The 37 °C incubation temperature was excluded from this two-factor model since only two of the five strains showed inhibition at this temperature.

In separate one-factor repeated-measures ANOVAs at each temperature, strains differed significantly ($P < 0.001$). Figures 2A–C show that a very similar pattern can be observed in the inhibitory effects of the strains at 20 and 25 °C, whereas at 30 °C, the pattern differs. However, strain SEFPRK2 no longer exhibits inhibition at this temperature. Strain SESK2 had the greatest inhibitory effect at all temperatures.

Figures 3A–E show the comparison of the temperatures by the different strains. For clarity, the figures also include temperatures at which no inhibitory effect of the strain was detected; however, only those temperatures at which an inhibitory effect was present were included in the one-factor repeated-measures ANOVA models and pairwise comparisons. The effect of the temperature was highly significant for all five strains ($P < 0.001$).

These results clearly indicate that inhibition of *L. innocua* by the tested endophytes is mostly influenced by the incubation temperature and that inhibition in certain strain–temperature combinations may fall below the threshold for the detection limit.

The data in Figs 2 and 3 show that the antagonistic capacity against *L. innocua* varies significantly between the isolates. Isolate “E” (SESK2) proved to be the most effective, producing

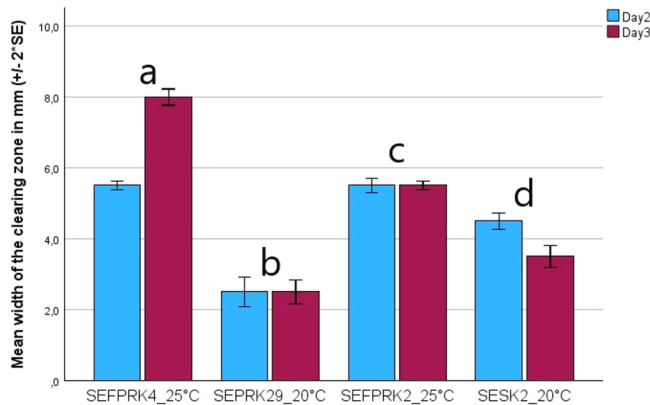


Fig. 1. Temperature dependence of inhibition for four putative endophytic bacteria (SEFPRK4, SEPRK29, SEFPRK2 and SESK2) taking into account the width of the cleaning zones produced against *E. coli* ATTC 8739 at either 20 or 25 °C. Cases with the same letters do not differ significantly according to Bonferroni’s post hoc test ($P > 0.05$)

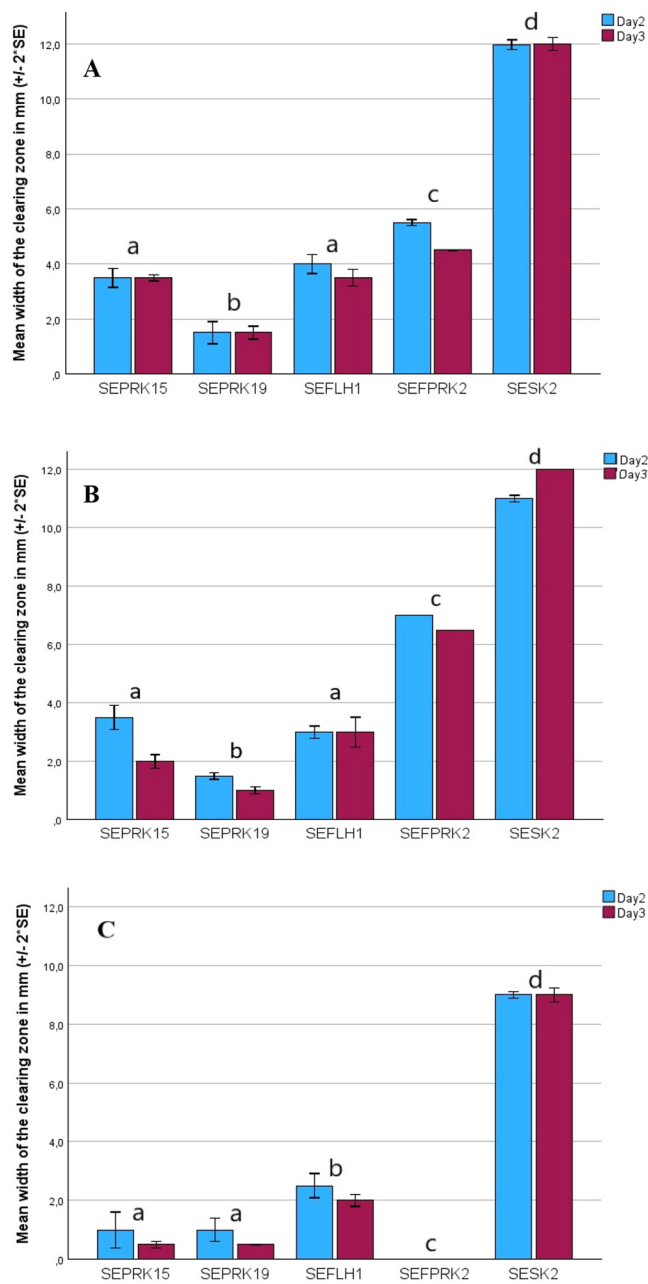


Fig. 2. Comparison of the clearing zones of five putative endophytic strains with inhibitory activity (SEPRK15, SEPRK19, SEFLH1, SEFPRK2 and SESK2) against *L. innocua* CCM 4030^T at three temperatures: (A) at 20 °C, (B) at 25 °C and (C) at 30 °C. Strains with the same letters do not differ significantly according to Bonferroni's post hoc test ($P > 0.05$)

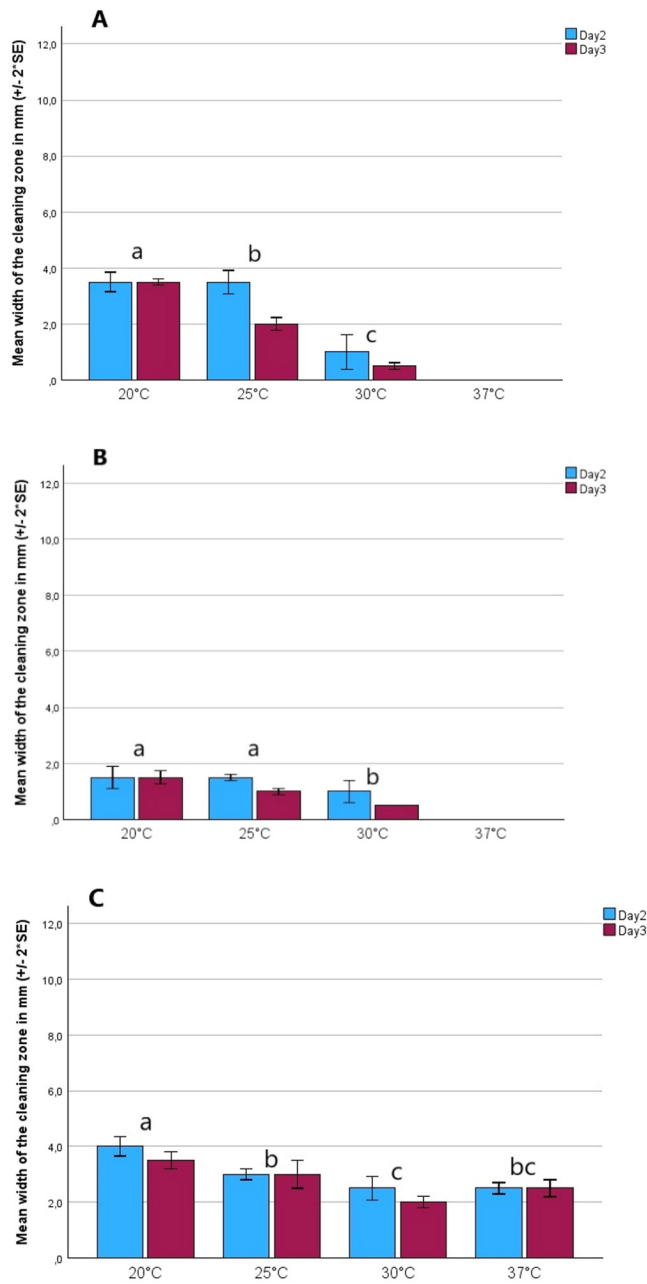


Fig. 3. The impact of temperature on the inhibitory effect of the five putative endophytic bacterial strains effective against *L. innocua* CCM 4030^T. Temperatures with the same letters do not differ significantly according to Bonferroni's post hoc test ($P > 0.05$)

(Figure 3 continued on next page)

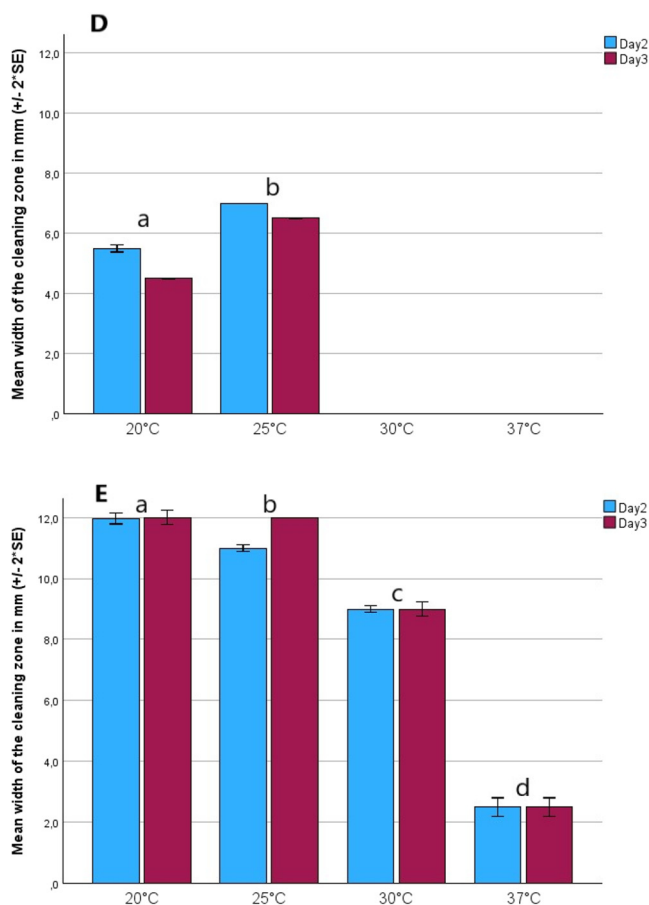


Fig. 3. Continued

a 12 mm clearing zone at 20 °C, which indicates a high production of antimicrobial compounds. Although isolates such as “B” (SEPRK19) and “C” (SEFLH1) were also active, their antagonistic zones were smaller, indicating different levels of efficacy (Figs 2–3B and 2–3C, respectively). Furthermore, isolates “C” (SEFLH1) and “E” (SESK2) showed a unique ability to maintain their inhibitory effect even at 37 °C (Figs 2–3C and 2–3E, respectively), making them more multi-faceted than the other strains. These differences highlight that the success of biocontrol does not only depend on the conditions, but also on the specific capabilities of the inhibitory strains themselves.

In summary, only a limited fraction of the investigated sweet pepper-derived putative endophytic bacteria exhibits robust antagonistic activity against *E. coli* and *L. innocua*, as certain natural environmental variables (e.g. incubation temperature and time) decrease the inhibitory potential below the detection limit. The SESK2 strain, which has been shown to inhibit both surrogate bacteria, is notable for its robustness because it demonstrated the ability to maintain

its significant inhibitory potential against *L. innocua* through the tested temperature range and incubation time. These results suggest that, for the purpose of biocontrol, it is essential to screen the robustness of the strains across varying environmental conditions.

Furthermore, the present research employed surrogate bacteria as a substitute for the target pathogens in the experimental settings. Kutter et al. (2006) conducted a study on the surface and endophytic colonisation ability of three *Listeria* strains. These strains belonged to the pathogenic *L. monocytogenes* and *L. ivanovii*, and the non-pathogenic *L. innocua*. It was found that all three strains exhibited a similar behaviour in that they colonised the surface of the barley root in a very similar number of cells. However, they were unable to colonise the inner tissues as endophytes. Barak et al. (2002) reported that the attachment of human pathogenic (O157:H7) and plant-derived *E. coli* strains to alfalfa sprouts was variable, with most of the latter adhering better to alfalfa sprouts than the pathogenic strain. Given the broad ecological niche of *E. coli* (Alm et al., 2011), it is essential to include *E. coli* strains of diverse origins, phenotypes and genotypes when evaluating and interpreting the results obtained with the selected surrogate(s) and assessing strain specificity.

Based on their consistent antagonistic performance, seven isolates (SEFPRK4, SEPRK29, SEFPRK2, SESK2, SEPRK15, SEPRK19, and SEFLH1) were selected for further evaluation of plant growth-promoting (PGP) traits, and their taxonomic identities were subsequently resolved.

3.2. Identification of the selected potential antagonistic endophytes

Molecular identification of the selected antagonistic endophytic strains was performed via sequencing of the 16S rRNA and *rpoB* genes. Using both the NCBI Nucleotide and the EzBioCloud databases for the 16S rRNA gene alignment, we concluded that SEFPRK4 belongs to *Enterobacter asburiae*, SEFLH1 to *Bacillus australimaris*, while the remaining five isolates cluster within the *Pseudomonas* genus (Table 2). The 16S rRNA gene-based identification failed to provide definitive species-level resolution within *Pseudomonas*, as multiple candidate species appeared with high degree of similarity in both databases. For each of the five *Pseudomonas* strains, NCBI BLAST returned multiple species-level hits with 100 % identity, and EzBioCloud similarly yielded more than one top-hit species above the 98.65 % cutoff – a threshold often applied for prokaryotic species delineation (Kim et al., 2014). To overcome this ambiguity, we sequenced the *rpoB* gene and used NCBI BLAST for sequence alignment. The combined evidence from both 16S rRNA and *rpoB* allowed us to assign distinct species identities to each isolate as follows: *P. grimontii* (SEPRK15), *P. alloputida* (SEPRK19), *P. koreensis* (SEPRK29), *P. palmensis* (SEFPRK2), and *P. protegens* (SESK2) (Table 2). Among these, the two strains (SEFPRK2 and SESK2) that demonstrated inhibitory activity against both surrogate pathogens, were identified as *P. palmensis* and *P. protegens*, respectively.

This dual-gene-based identification approach underscores a known limitation of the 16S rDNA sequence analysis: while it is useful for genus-level identification, its slow evolutionary rate and high conservation often render it insufficient for reliable species-level discrimination within closely related taxa, such as the *Pseudomonas* genus. In contrast, protein-coding house-keeping genes like *rpoB*, *gyrB*, and *rpoD* evolved more rapidly and thus often provide higher resolution for intra-genus phylogenetics and correct speciation (Ait Tayeb et al., 2005; Yamamoto et al., 2000). Moreover, databases differ in their approaches: NCBI GenBank offers a

Table 2. Identification of the antagonistic putative endophytic bacterial strains by BLAST analysis of the 16S rRNA and the *rpoB* genes. Accepted species names are in bold

No.	Strain ID	16S rRNA gene sequences				<i>rpoB</i> gene sequences			
		GenBank Acc. No.	Identification using NCBI Nucleotide database		Identification using EzBioCloud database		GenBank Acc. No.	Identification using NCBI Nucleotide database	
			Species name	Similarity %	Species names	Similarity %		Species names	Similarity %
2.	SEPRK15	KU605704	<i>P. grimontii</i>	100	<i>P. grimontii</i>	100	KX958162	<i>P. grimontii</i>	100
			<i>P. fluorescens</i>	100	<i>P. rhodesiae</i>	99.9		<i>P. marginalis</i>	98.63
			<i>P. rhodesiae</i>	100					
3.	SEPRK19	KU605703	<i>P. alloputida</i>	100	<i>P. alloputida</i>	100	KX958174	<i>P. putida</i>	99.76
			<i>P. fluorescens</i>	100	<i>P. hunanensis</i>	100		<i>P. kurugenalensis</i>	99.76
			<i>P. hunanensis</i>	100	<i>P. promysalinigenes</i>	100		<i>P. hunanensis</i>	99.64
			<i>P. juntendi</i>	100				<i>P. taiwanensis</i>	99.64
			<i>P. monteilii</i>	100				<i>P. alloputida</i>	98.81
			<i>P. plecoglossicida</i>	100					
			<i>P. putida</i>	100					
5.	SEFPRK4	KU605731	<i>Enterobacter asburiae</i>	99.92	<i>E. asburiae</i>	99.61	–	n.d.	n.d.
11.	SEFLH1	KU605706	<i>Bacillus australimaris</i>	100	<i>B. australimaris</i>	100	–	n.d.	n.d.
15.	SEPRK29	KU605700	<i>P. koreensis</i>	100	<i>P. koreensis</i>	100	KX958165	<i>P. fluorescens</i>	99.64
			<i>P. fluorescens</i>	100	<i>P. iranensis</i>	99.8		<i>P. putida</i>	99.28
			<i>P. putida</i>	100				<i>P. koreensis</i>	99.04
			<i>P. umsongensis</i>	100					
31.	SEFPRK2	KU605677	<i>P. palmensis</i>	100	<i>P. palmensis</i>	100	KX958170	<i>P. qingdaonensis</i> ^a	99.23
			<i>P. qingdaonensis</i> ^a	100	<i>P. akappageensis</i>	99.88			
			<i>P. putida</i>	100	<i>P. defluvii</i>	99.88			
			<i>P. japonica</i>	100					
			<i>P. kilonensis</i>	100					
			<i>P. brassicae</i>	100					
			<i>P. defluvii</i>	100					

(continued)

Table 2. Continued

No.	Strain ID	16S rRNA gene sequences					<i>rpoB</i> gene sequences		
		GenBank Acc. No.	Identification using NCBI Nucleotide database		Identification using EzBioCloud database		GenBank Acc. No.	Identification using NCBI Nucleotide database	
			Species name	Similarity %	Species names	Similarity %		Species names	Similarity %
35.	SESK2	KU605698	<i>P. protegens</i>	100	<i>P. protegens</i>	100	KX958159	<i>P. protegens</i>	100
			<i>P. chlororaphis</i>	100	<i>P. saponiphila</i>	99.40			
			<i>P. danubii</i>	100	<i>P. sesami</i>	99.40			
			<i>P. fluorescens</i>	100					
			<i>P. idahonensis</i>	100					

SEPRK: main root of hydroponic Kárpia cultivar.

SEFPRK: main root of soil-grown Kárpia cultivar.

SEFLH: leaf of soil-grown Hó cultivar.

SESK: steam of hydroponic Kárpia cultivar.

P.: *Pseudomonas*.

n.d.: not determined.

^a basonym of *P. palmensis*.

broad, annotated repository of DNA sequences across many organisms, though it may include misannotations or outdated nomenclature. In contrast, EzBioCloud provides a comprehensive, quality-controlled genome database encompassing only type strains with taxonomic identification at genus, species and subspecies levels (Chalita et al., 2024). The EzBioCloud database is regularly curated and updated by DSMZ (German Collection of Microorganisms and Cell Cultures), enhancing its reliability for taxonomic assignments. However, its utility is constrained by the inherent resolution limitation of the 16S rDNA. Therefore, the use of both databases in parallel improves confidence in species-level identifications (Park et al., 2012).

The results of species-level identification based on the *rpoB* gene did not always corroborate identification based on the 16S rDNA. This may be due to the varying evolutionary rates of the two markers or the different quality and coverage of the reference databases. The results do not necessarily show incorrect identification; they highlight the uncertainty surrounding the taxonomic status of the examined strains. Therefore, *rpoB* did not compromise the results; it provided a more rigorous, higher-resolution check, revealing the uncertainty of species-level classification.

In the context of biocontrol and functional screening, accurate species identification is not merely academic: the metabolic and antagonistic potential can vary significantly even between closely related *Pseudomonas* species. By applying the combined 16S rRNA + *rpoB* strategy, we can improve taxonomic resolution, enabling us to link inhibitory phenotypes to a given species. Future work may also benefit from multilocus sequence analysis (MLSA) or even whole-genome sequencing (WGS) to further validate species identities and to explore gene clusters responsible for antagonistic metabolite production.

3.3. Determination of the plant growth-promoting activity (PGPA) of the selected antagonistic putative endophytic strains

The direct and indirect plant growth promotion (PGP) mechanisms of the selected seven antagonistic endophytic strains were evaluated, focusing on macro- and micronutrient solubilisation (phosphorus and zinc), IAA production, and cellulase activity as a proxy for plant colonisation potential (Table 3). All strains displayed at least one PGP-associated trait: zinc solubilisation was the most common, while IAA production was unique. Four strains were found to harbour only one PGP trait, two strains exhibited two traits, and only one strain exhibited three traits. It was observed that none of the strains possessed all four traits, and no strain was found to be negative for all the traits that were examined. Among the seven strains, *P. protegens* SESK2 emerged as the most functionally versatile isolate: it solubilised phosphate and zinc and produced cellulase (Fig. 4A–D). Meanwhile, *E. asburiae* SEFPRK4 was unique in producing IAA as its sole PGP trait. However, it has to be stated that the inhibitory halos and solubilisation zones were measured using a standardised millimeter-scale ruler. Photographic documentation provides qualitative visual evidence of the activity, whereas the quantitative data presented in Table 3 reflect the exact measurements of the total zone diameter, including the diffuse margins which might show reduced visibility in photographic representation due to limitations in optical contrast.

The dual antagonistic activity and growth-promoting capacity of *P. protegens* SESK2 aligns with published reports: *P. protegens* strains are producing biocontrol compounds such as 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin, which contribute to the suppression of

Table 3. PGP traits of the antagonistic potential endophytic bacteria. PGP activities were characterised by establishment of typical zones around the macrocolonies in indicator culture media as described in 2.4. Widths of the zones are indicated in mm ± SD

No.	Strain ID	Species	IAA production (mm)	Phosphate solubilisation (mm)	Zinc solubilisation (mm)	Cellulose degradation (mm)
2.	SEPRK15 ^a	<i>P. grimontii</i>	–	–	20 ± 2	–
3.	SEPRK19 ^a	<i>P. alloputida</i>	–	–	18 ± 2	–
5.	SEFPRK4 ^b	<i>E. asburiae</i>	23 ± 1	–	–	–
11.	SEFLH1 ^a	<i>B. australimaris</i>	–	–	28 ± 3	6 ± 1
15.	SEPRK29 ^b	<i>P. koreensis</i>	–	2 ± 0	28 ± 0	–
31.	SEFPRK2 ^{a,b}	<i>P. palmensis</i>	–	–	15 ± 2	–
35.	SESK2 ^{a,b}	<i>P. protegens</i>	–	3 ± 1	34 ± 1	7 ± 0

SEPRK: main root of hydroponic Kárpia cultivar.
SEFPRK: main root of soil-grown Kárpia cultivar.
SEFLH: leaf of soil-grown Hó cultivar.
SESK: steam of hydroponic Kárpia cultivar.

P.: *Pseudomonas*.
E.: *Enterobacter*.
B.: *Bacillus*.

^ainhibitory effect on *L. innocua*.
^binhibitory effect on *E. coli*.

plant pathogens and support beneficial plant–microbe interactions (Ramette et al., 2011). The ability of *P. protegens* SESK2 to solubilise phosphate and zinc, combined with cellulolytic activity, further strengthens its candidacy as a multifunctional biocontrol + PGP agent. Meanwhile, *P. palmensis* SEFPRK2, another strain that inhibited both surrogate pathogens, displayed only zinc solubilisation ability among the tested traits. While *P. palmensis* is less documented in PGP contexts, previous studies have reported its capacity for iron nutritional support and IAA production, siderophore synthesis, and phosphate solubilisation (Gutierrez-Albanchez et al., 2021). It is noteworthy that *E. asburiae* SEFPRK4 was the only isolate producing IAA among the selected strains but not any other tested traits could be detected. The published *E. asburiae* PGP strains have been shown to produce ammonia and siderophores beside the IAA, solubilise phosphate, and mobilise zinc — features consistent with roles in plant growth support (Abraham and Silambarasan, 2015). *E. asburiae* has also been implicated in antagonistic activity against *E. coli* O157:H7, when introduced into cantaloupe melon (Tran et al., 2024).

Overall, our findings demonstrate that antagonistic endophytes isolated from sweet pepper exhibit complementary PGP traits, although multifunctionality appears to be uncommon. The combined presence of strong inhibitory activity and growth-promoting potential in *P. protegens* SESK2 highlights its promise as a candidate for biocontrol applications. Elucidating the specific compounds responsible for the observed antagonism and verifying the efficacy of the identified strains *in vivo* will be essential to confirm their involvement in food safety engineering.

Future studies for biofertiliser applicability should extend trait profiling to include additional PGP mechanisms such as siderophore production, ACC deaminase activity, and the biosynthesis

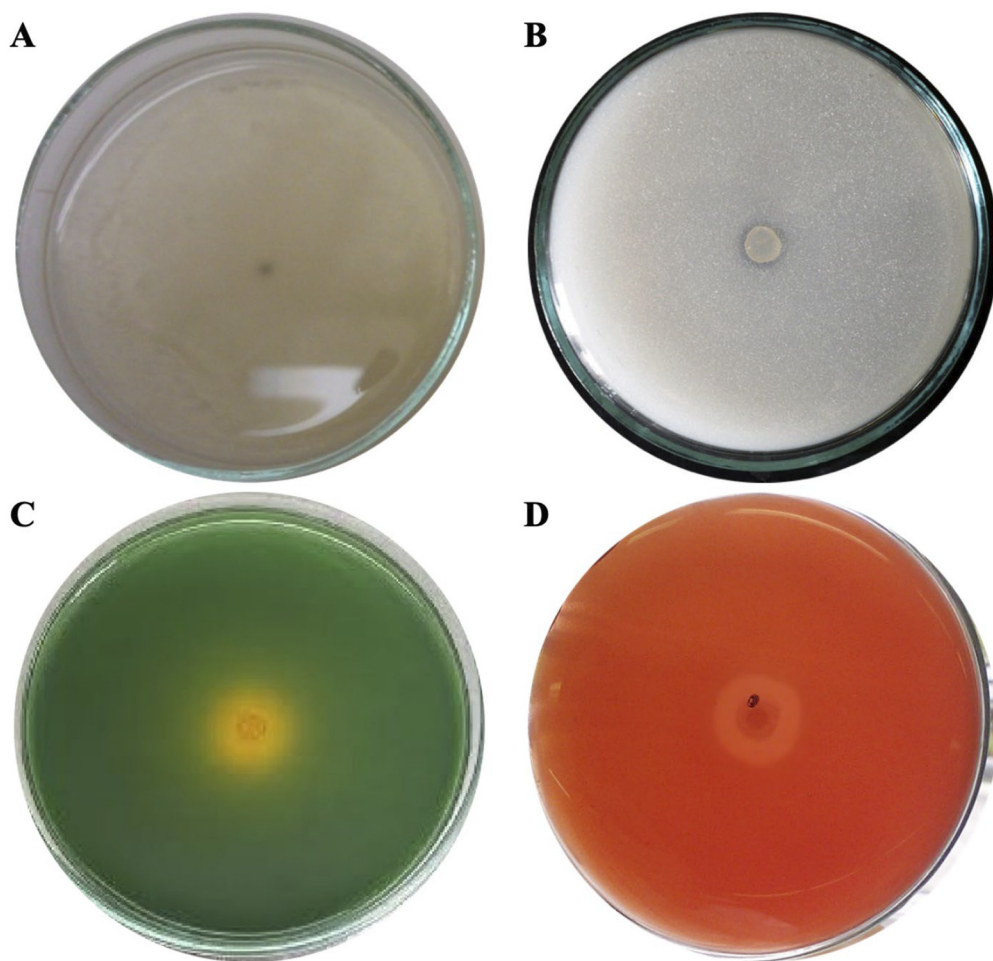


Fig. 4. Illustration of the PGP properties of the *P. protegens* SESK2 strain. A: indole-3-acetic acid (IAA) production (negative); B: phosphate solubilisation (positive); C: zinc solubilisation (positive); D: cellulase activity (positive)

of antimicrobial secondary metabolites, followed by validation of these functions *in planta* under greenhouse conditions.

Despite the significant antagonistic effects detected in our tests, there are some limitations that must be considered. The results obtained are based on *in vitro* assays, which means the actual performance of these isolates might vary in real food products due to certain environmental factors such as pH, salt content, or storage temperature. Prior to the implementation of any practical applications, further studies are required to ascertain the safety and genome stability of the strains. If confirmed, these strains, particularly those remaining stable at elevated temperatures, have the potential to be useful within the food industry. Their potential to work as protective cultures in dairy or meat products could be considered a new way of biocontrol

activity for preventing contamination by *Listeria*. Furthermore, the inhibitory substances can act as components of sanitisation agents, with the aim of reducing development of pathogen's biofilms on equipment surfaces. This approach offers a natural alternative to chemical preservatives.

4. CONCLUSIONS

The putative bacterial endophytes isolated from sweet pepper displayed diverse plant growth-promoting traits and *in vitro* inhibitory activity against selected surrogates of foodborne pathogens. These results demonstrate the potential of sweet pepper-associated putative endophytic bacteria for biocontrol purposes. We identified *P. protegens* SESK2 as the most promising strain within the tested endophytes. Nevertheless, the current findings are based solely on laboratory experiments, and no conclusions can yet be drawn regarding the performance of these bacteria under plant cultivation conditions or in real food systems. Further studies, including *in planta* assessments and evaluation under greenhouse and field conditions, are necessary to assess their practical applicability.

AI use disclosure: ChatGPT and DeepL were used solely for language editing and improving the clarity of the manuscript. All scientific content, analyses, and conclusions are the original work of the authors.

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SUPPLEMENTARY MATERIAL

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