



Unraveling the ecological impact of a bacterial biocontrol agent applied to melon plants: insights into phenotypic, biochemical, and microbiota changes affected by *Frateuria defendens*

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Accepted: 23 January 2025 / Published online: 13 February 2025
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Abstract The increasing use of bacterial biocontrol agents in agriculture raises concerns about their potential impact not only on the environment of infected plants but also on that of healthy plants. This study investigated the effects of introducing the endophytic bacterium *Frateuria defendens*, a potential biocontrol agent against phytoplasma diseases, on melon (*Cucumis melo*) as a model plant. Different isolates of *F. defendens* were screened for their *in vitro* and *in planta* antimicrobial activity, as well as their ability to colonize and influence the plant microbiome. Only one isolate, designated KT11, successfully colonized the melon shoot and was found in the plant sap. The antimicrobial activity of this isolate was retained in the plant sap and was confirmed in a biological test on *Spiroplasma melliferum*. The presence of KT11 in the plant tissue induced distinct shifts in the overall structure and diversity of the endophytic microbial populations. These significant findings emphasize the need for a comprehensive understanding of the ecological implications of introducing exogenous

microbes into plant systems. This study contributes to the growing body of knowledge on the intricate interactions between plants, their microbiomes, and introduced biocontrol agents, towards performing sustainable agricultural practices.

Keywords *Frateuria defendens* · Melon microbiome · Bacterial biocontrol · Microbial ecology

Introduction

The increasing utilization of microbial inoculants and their secreted metabolites as a biological control solution in modern agricultural practices (Santos et al., 2019) emphasizes a significant gap of knowledge concerning the implications of introducing exogenous bacterial species into plants' natural habitats. Given that agricultural fields treated with microbial biocontrol agents contain both infected and healthy plants, questions arise regarding the potential effects of these agents on uninfected plants. From an environmental perspective, a plant can be seen as an ecological niche for various microorganisms that inhabit its tissues. Therefore, inoculation of the plant and its surroundings with exogenous beneficial bacteria can significantly alter the diversity and structure of the microbial community (Senthilkumar et al., 2011; Welmillage et al., 2021), thereby may affect various plant traits (Yurgel et al., 2022). These changes can

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-025-03012-5>.

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be particularly pronounced when the biocontrol agent is an endophyte. Endophytic bacteria have the unique ability to colonize the internal tissues of their host plant without causing any phytotoxic effects (Pacífico et al., 2019). They can establish a wide range of relationships with their plant hosts, from transient associations to more mutualistic symbioses. These relationships can help mitigate biotic and abiotic stresses that hinder plant growth and development (Mercado-Blanco & Lugtenberg, 2014; Rosenblueth & Martínez-Romero, 2006). The mechanisms by which endophytic bacteria reduce biotic stress, caused by phytopathogenic microorganisms, include competition, antibiotic secretion, and the enhancement of the plant's innate immune system (Ryan et al., 2008). These processes can directly or indirectly suppress the growth and virulence of plant pathogens, thereby promoting the health and productivity of the infected plant.

One significant advantage of utilizing endophytic bacteria as biological control agents is their ability to enter the plant's internal tissues and subsequently spread systemically throughout the host via the vascular system and other transport mechanisms (Hardoim et al., 2008; Rosenblueth & Martínez-Romero, 2006; Vandana et al., 2021). This endophytic colonization enables the bacterial biocontrol agent to combat phytopathogens at the site of infection, offering a more targeted and effective disease control than epiphytic bacteria (colonizing plant's surface).

Fratureia defendens (Lidor et al., 2019) is a newly discovered endophytic bacterium that was originally isolated from the phloem-feeding planthopper insect *Hyalesthes obsoletus* (Iasur-Kruh et al., 2018). These cells are gram-negative, aerobic, motile, and appear as small rod-shaped structures measuring 0.2–0.3 µm wide and 1.0–1.3 µm long (Lidor et al., 2019). *F. defendens* penetrates plants via roots or leaf stomata, and it can colonize the xylem and phloem of various plant species (such as cotton, melon, grapevines, etc.) without causing any phytotoxic effects (Lidor et al., 2018). Furthermore, this bacterium is a potential biocontrol agent against yellows disease, attributed to *Candidatus* Phytoplasma bacteria of the Mollicutes class (Gonella et al., 2019; Naor et al., 2023). Since phytoplasma cannot be cultivated in axenic culture, it is customary to use the bacterium *Spiroplasma melliferum* (also belongs to the Mollicutes class) as a model bacterium (Iasur-Kruh et al., 2018). Indeed, *F. defendens* showed an ability to suppress *S. melliferum*

activity *in vitro* and reduce phytoplasma symptoms *in planta* both in grapevines and periwinkle (Iasur-Kruh et al., 2018; Naor et al., 2019). In addition, when applied in vineyard, *F. defendens* reduced yellows disease symptoms in infected vines under field conditions (Naor et al., 2019). In a previous study, we showed that *F. defendens* secrete secondary metabolites that inhibit the growth of mollicute bacteria, and it was suggested that these secreted metabolites, combined with the bacterium localization *in planta*, are the main mechanism that results in the reduction of disease symptoms in phytoplasma-infected plants (Naama-Amar et al., 2020). However, comparing the efficiency between strains of *F. defendens* has never been studied, nor has the impact of *F. defendens* on the plant microbiome. In the current study, we utilized several *F. defendens* isolates to investigate the plant-bacterium interaction on the entire host plant biosphere, focusing on their effects on plant sap. To facilitate this investigation, we selected healthy melon plants as hosts due to their rich sap production, which is easily extractable and examinable. This study's objectives were to compare the inhibitory activity among isolates of *F. defendens* and examine the effects of selected isolates on the antibacterial activity of the plant's sap and its endophytic community composition.

Material and methods

Bacterial strains and growth conditions

The bacterial strains of *F. defendens* were obtained from Dr. Einat Zchori-Fein at the ARO research center. Different *F. defendens* isolates obtained from the guts of the insect *Hyalesthes obsoletus*, collected previously and stored in –20 °C were used for the study. The strains were designated as KT11, KT14–24 & LH44 (Table 1). To initiate the bacterial starter of *F. defendens*, 100 µL from glycerol stock of each isolate were inoculated into 20 mL of LB culture medium (Table S1) and incubated at 28 °C for 48 h. All strains were plated on CV agar to assess the presence of *F. defendens* by viable count (Table S1, Iasur-Kruh et al., 2018). Subsequently, 5 mL of the starter culture for each isolate was transferred separately into 20 mL of S medium. (Table S1, Trachtenberg & Gilad, 2001) for 10-day incubation at 28 °C on a shaker to enable the isolate to secrete inhibitory metabolites.

Table 1 The inhibitory effect of filtrates of different isolates of *F. defendens* on *S. melliferum* growth¹. The percentage of *S. melliferum* growth inhibition was calculated by comparing the growth measurement in the presence of different *F. defendens*' filtrates to that of the positive control at a specific time point

Isolate	Isolate origin		% Inhibition after 3 days incubation	Time (h) to 75% inhibition threshold
	Year	Site		
KT11	Fall, 2013	Kidmat Tsvi	75±6.3	144
KT14	Fall, 2020	Kidmat Tsvi	100±1	144
KT15	Fall, 2020	Kidmat Tsvi	100±1.3	>168
KT16	Fall, 2020	Kidmat Tsvi	94±3.8	48
KT17	Fall, 2020	Kidmat Tsvi	78±3.8	48
KT18	Fall, 2020	Kidmat Tsvi	25±5.7	64
KT19	Fall, 2020	Kidmat Tsvi	100±6	66
KT20	Fall, 2020	Kidmat Tsvi	100±22	40
KT21	Fall, 2020	Kidmat Tsvi	100±5.5	64
KT22	Fall, 2020	Kidmat Tsvi	100±6.6	68
KT23	Fall, 2020	Kidmat Tsvi	100±9.7	120
KT24	Fall, 2020	Kidmat Tsvi	100±13	120
LH44	Fall, 2020	Neve Ya'ar	100±19	56

¹*S. melliferum* cultures were obtained from Professor Shlomo Trachtenberg's laboratory at the Hebrew University of Jerusalem. *S. melliferum* starter was established by cultivating it in an S medium at 28 °C for five days. This medium was formulated to simulate the concentration and composition of plant sap, incorporating sugars, organic compounds, and diverse macromolecules, creating high osmotic pressure (Serra-Soriano et al., 2015).

Evaluation of the inhibitory activity of *F. defendens* strains

Experimental design The inhibitory effect of *F. defendens* isolates was assessed *in vitro* by comparing the inhibition activity of the isolates' filtrate. The working hypothesis assumed that secondary metabolites are secreted into the growth medium and retain the inhibition activity (Naama-Amar et al., 2020). The filtration of 13 isolates was tested at a single time point after three days of incubation and by continuous monitoring for seven days (As detailed above). Each treatment was performed in five replicates.

***F. defendens* filtrate separation** To obtain a filtrate containing secreted secondary metabolites, the cell

culture of each isolate was grown for ten days. Subsequently, the culture was centrifuged for 10 minutes at 5000 rpm at room temperature, and the supernatant was filtered through a 0.22-µm filter (FPV, JET BIO-FIL, Spain) to obtain cell-free supernatant.

Filtrate inhibition test Each filtrate obtained from different *F. defendens* isolates was assessed following a previously described protocol (Iasur-Kruh et al., 2018). Briefly, 0.5 mL of the cell-free supernatant of each strain was mixed with 0.5 mL of S medium (Table S1) in an Eppendorf tube, adding five µL of *S. melliferum* cells (approximately 8×10^5 cells) to the mixture and was incubated for five days at 28 °C. As a positive control, 1 mL of S medium supplemented with five µL of *S. melliferum* was used, while sterile S medium without bacterial cells was a negative control. At the end of this stage, the inhibitory effect was assessed using a 96-well plate assay: Five µL from each Eppendorf tube were added to 200 µL of S-red medium (Table S1) in each well and incubated at 28 °C. *S. melliferum* growth was monitored by measuring OD values: red signified inhibition of bacterial growth, while yellow indicated bacterial growth. The inhibitory activity was evaluated using two methods: a. Single time point measurement: OD_{595nm} was recorded after three days of incubation; b. Continuous monitoring: A CLARIOstar plate reader system was used to track OD_{450nm} continuously throughout the incubation period. In both tests, the inhibitory level was calculated as the relative change in yellow color compared to a positive control (%). The inhibition period was calculated as time to complete color change (hours). To compare between strains, the time to 75% inhibition was set as a threshold. These two methods enabled us to distinguish between the inhibition levels of different strains over time. The test was performed in 5 replicates for each treatment. Inhibition was calculated as percentage growth of *S. melliferum* compared to growth in respective positive controls. Inhibition (%) = $(1 - (\text{OD}_{595\text{nm}} \text{ with filtrate} / \text{OD}_{595\text{nm}} \text{ in positive control})) \times 100$.

Evaluation of *F. defendens* strain KT11 on plant sap and endophytic community composition

Experimental design The effect of KT11 isolate on melon plants was examined by investigating both plant sap and DNA extracted from plant

tissues. To explore the changes in sap antimicrobial traits, 450 µL of plant sap was analyzed in a single time point inhibitory test (see below). The working hypothesis posits that the isolate inhabits the plant's vascular tissues and secretes its metabolites directly into the sap, even in the absence of pathogenic bacteria. As a result, KT11 isolate presence can alter the microbiome of a healthy plant. Therefore, an NGS analysis was done on KT11-applied and control plant tissues.

Plant material and growth conditions The *in-planta* experiments used young melon plantlets (14 days old) of the variety Nay Yizael, provided by Dr. Amit Gorss's laboratory, Volcani Center-Agricultural Research Center at Neve Yaar. The seeds were sown in a horticultural soil mixture, Garden Mix Series, at 25 °C under long-day conditions (16 h light/8 h dark) for two weeks before treatment.

Application of *F. defendens* to melon plants The three isolates exhibiting prolonged inhibitory activity, KT11, KT14, and KT15, were used to study the ability to enter and reside in vascular plant tissue (Table 1). Cell suspension of each isolate, containing approximately 2×10^7 cells/mL and supplemented with 0.1% Tween 20 in DDW, was sprayed on two-week-old melon plants in 30 replicates. Fifteen plants were sprayed with LB liquid medium supplemented with 0.1% Tween 20 as a control, and 15 plants were sprayed with DDW as another control for the entire process. The presence and identity of *F. defendens* isolates in plant tissues were determined using classical microbiological methods and specific PCR. In addition, the bacterium's impact on plant growth (length and weight) was examined five and ten days after application. The experiment was repeated three times.

Plant sap extraction Plants were sampled for sap extraction five- and ten-days post the *F. defendens* application. Each plant was surface sterilized separately by immersing the plant parts in 70% ethanol for 30 seconds, a 2-minute immersion in NaOCl 0.6%, and two 10 min rinses in DDW. Subsequently, plant sap extraction was performed separately from the stem of each plant. The stem was cut into 2 cm pieces under sterile conditions, and from each piece, plant sap was extracted by inserting the tip of a small

pipette into the bottom part of each stem piece, primarily aiming to obtain pure phloem sap. However, the pressure exerted on the stem by fingers resulted in a mixed sap containing both phloem and xylem saps, hence called plant sap. Approximately 500 µL of mixed sap were collected and pooled from three treated plants. Sap collected and pooled from three non-inoculated plants served as a control.

Viable counts *F. defendens* in plant sap Fifty µL of plant sap collected five and ten days post-treatment were plated on CV agar to assess the presence of *F. defendens* by viable count. Colony identification was confirmed by PCR analyses using specific primers: PCR mixture of each tube contained 10 µL of DDW, 12 µL of Kodaq 2x PCR Master Mix, and 1 µL of each primer (5 picomoles): F- CTCTGTGGGTGGCGA GTGGC, R- ACCGTCAGTTCGCCGGG (Naor et al., 2019). The reaction conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 64 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes.

Inhibitory effect of plant sap on *in vitro* growth of *S. melliferum* Plant sap was collected from both control and KT11-treated plants (as described previously). To prevent contamination, the sap samples were heat-treated by boiling for 5 minutes. Subsequently, 55 µL of the treated sap was added to 200 µL of S-red medium in a 96-well plate. The medium was then inoculated with 1 µL of *S. melliferum* culture and incubated at 28 °C for five days. This experiment was performed with five biological replicates, with each replicate consisting of pooled sap from three plants.

DNA extraction from plant Plant tissues were surface sterilized as detailed above, and the shoots and the roots were separated (Table S2). Total DNA was extracted using a Plant/Fungi DNA Isolation kit (Plant/Fungi DNA Isolation kit, Norogen Biotek Corp., Thorold, Canada). In the first stage, the plant tissues were homogenized with lysis buffer in a 1:2 ratio using a sterilized mortar and pestle. All following steps were according to the manufacturer's instructions. DNA quantity and quality were examined using a NanoDrop spectrophotometer (NanoDrop 2000/2000c; Thermo Fisher Scientific,

Waltham, MA). The extracted DNA was used as template for a *F. defendens*-specific PCR assay, as described above. This was done in order to confirm *F. defendens* presence or absence in treated plants as well as in the non-treated plant controls.

Determination of endophytic bacterial community composition In order to examine the effect of application of the *F. defendens* isolate on melon endophytic communities, amplicon sequencing of 16S rRNA gene was performed. The extracted DNA (See above) was used for Next-Generation Sequencing (NGS) by amplicon sequencing: Genomic DNA was PCR amplified with primers CS1_520F and CS2_799R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCA GCMGCCGCGGTAA and GGACTACNVGGG TWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes, with reduced affinity to plastid sequence (Moroenyane et al., 2021). The reaction conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 29 cycles of denaturation at 94 °C for 30 seconds, annealing at 62 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes. Library preparation, pooling, and sequencing were performed at the Genome Research Core (GRC) within the Research Resources Center (RRC) at the University of Illinois at Chicago (UIC) as described previously (Naqib et al., 2018). The re-pooled libraries, with a 15% phiX spike-in, were loaded onto a Miniseq flow cell, and sequenced (2×153 paired-end reads). Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Raw sequence data was processed using the Dada2 pipeline (Callahan et al., 2016) using the R package ‘dada2’ (version 1.28.0). Sequences were filtered and trimmed for quality using the ‘filterAndTrim’ command with the parameters maxN set to zero, maxEE set to 2, and trimLeft set to 15 bp. The truncLen was set to 153 bp and 150 bp for the forward and reverse reads, respectively. The sequence error estimation model was calculated using the ‘learnErrors’ option using default parameters. The dada2 algorithm for error correction was applied with the ‘dada’ command using default parameters. Sequences were merged using the ‘mergePairs’

command with minimum overlap set at 8 bp. Following, suspected chimera was detected and removed using the command ‘removeBimeraDenovo’. A count table including each ASV (Amplicon Sequence Variant) in each sample was then produced. To obtain a taxonomic assignment for each ASV, each ASV sequence was aligned to the ARB-Silva small subunit rRNA database (version Silva_nr_138.1) using the command ‘assignTaxonomy’ with default parameters, but minBoot set at 80%. A count table, adjoined with taxonomic assignment for each ASV was produced. All sequences with length < 249 bases or length > 265 bases were removed. In addition, ASVs of non-bacterial origin were filtered out, including chloroplast, mitochondria, Archaea, and unclassified origin. In total 1,315,295 reads and 1020 unique ASVs were obtained.

Statistical analysis

Viable counts of *F. defendens* (log 10 transformed) and levels of inhibitory activity in plant sap were compared between treatment and control using the non-parametric aligned rank-transform for analysis of variance (ART-ANOVA, Wobbrock et al., 2011) method. This was done with R package ‘ARTool’ (version 0.11.1). The model included treatment, time point and their interaction. Differences were considered significant for $P < 0.05$.

In order to compare the composition of plant endophytic communities, count data was normalized using the cumulative sums scaling (CSS) method (Paulson et al., 2013). The contribution of treatment, time point, and their interaction to variation in endophytic community composition were tested using permutational analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities. This was done with function ‘adonis2’ in R package ‘vegan’ with 1000 permutations. Non-metric multidimensional scaling (NMDS) was calculated for 2 dimensions based on Bray-Curtis dissimilarities. In order to compare relative abundances of specific genera, linear decomposition model analysis of effect size (LEfSe) was applied using command ‘run_lefse’ in R package ‘microbiomeMarker’ (version 1.6.0). Differences were considered significant for $P < 0.05$ and LDA effect size > 2.

Results and discussion

The current study surveyed isolates of *F. defendens* exhibiting potent inhibitory activity. Selected isolates were then introduced as exogenous endophytes into melon plants to investigate the impact on the plant phenotype, the antibacterial properties of the plant sap, and the composition of the plant's endophytic community.

The inhibitory activity of *F. defendens* strains filtrate

Given that bacteria secrete inhibitory substances in a strain-specific manner (Bertuzzi et al., 2022; Hashem et al., 2019; Islam et al., 2012; Park & Andam, 2019), the initial step of the current research was the identification of the optimal isolate for this study. Several isolates were obtained from insects gathered from the same site at the same season from which the original *F. defendens* (KT11 strain) was isolated (Table 1). These strains were isolated in 2020, seven years after KT11 was isolated. All isolates were obtained from *Hyalesthes obsoletus* specimens, the primary insect vector that transmits *Phytoplasma*—the causal phytopathogen of yellows disease in grapevine in Israel (Sharon et al., 2015). The insect specimens were captured from *Vitex Agnus cactus* plants located in a rich grapevine-growing area in the northeast region of Israel during the insect flight period. Previous studies have shown that KT11 produces secondary metabolites that reduce yellow disease symptoms *in planta* on the one hand and inhibit model Mollicute (*Spiroplasma*) on the other (Naama-Amar et al., 2022; Naor et al., 2019). Hence, the inhibitory effect of different isolates' secretions on *Spiroplasma* was examined. After three days of incubation, nine out of 13 isolates showed strong inhibitory activity, whereas the other four strains displayed inhibition ranging from 25% to 94%. However, only KT-14, KT-15, and the original isolate—KT11, sustained a strong inhibition activity of 75% or higher after prolonged incubation of at least six days; these isolates were chosen for further examination *in planta*.

Various techniques exist for applying endophytic bacteria as biocontrol agents, depending upon the plant type and the endophyte strain, including seed (Rana et al., 2019), soil (Munif et al., 2013), and foliar (Fu et al., 2020) applications. A previous field trial has shown that applying KT11 by foliar spraying

reduced *Phytoplasma* symptoms in the vineyard (Naor et al., 2019). When applied through leaf spraying, endophytic bacteria enter the phyllosphere via plant stomata or enzymatic breakdown of the plant wall. They form biofilms, creating a conducive micro-environment for endophyte colonization, and distribute by moving within the apoplast or vascular tubes (Liu et al., 2017; Pinski et al., 2019). While some endophytic bacteria remain near their entry point, others spread systemically throughout the plant (Pinski et al., 2019). Indeed, *F. defendens* possesses flagella, possibly facilitating its movement and settlement in plant tissues (Lidor et al., 2018). In the current study, out of the three isolates applied via foliar spraying, only KT11 penetrated and colonized the inner tissues of melons, as confirmed by PCR analysis (Table S1). Furthermore, KT11 could colonize the melon's shoot but not the root and inhabit it for at least ten days without causing changes in the plant morphology (Table S2), conforming endophytic interaction.

These findings emphasize the importance of adopting a strain-specific approach in the application of biocontrol agents, as the functional properties of bacteria can vary significantly even within the same species (Medina et al., 2007). The observed isolate-specific differences in inhibitory activities and interactions with the plant host align with characteristic differences of other bacterium strains. For example, numerous studies have demonstrated strain-specific production of diverse secondary metabolites by *Bacillus subtilis* bacteria (Bertuzzi et al., 2022; Hashem et al., 2019; Islam et al., 2012) and different strains of *Streptomyces rimosus* exhibited distinct antibiotic production profiles (Park & Andam, 2019). Furthermore, other endophytes show a strain-specific match between endophyte strains and the plant hosts. Different strains of the same endophyte species may have varying abilities to interact with different plant genotypes, depending on their strain-specific capacities for chemotaxis, enzyme secretion, and biofilm formation (Brader et al., 2014; Lalar et al., 2022; Pinski et al., 2019; Rosenblueth & Martínez-Romero, 2006).

The presence of KT11 in melon sap and the effect on its inhibitory activity

The current study found that KT11 is present in the plant's sap when applied via foliar spraying on melons (Fig. 1). The sap contains two main types

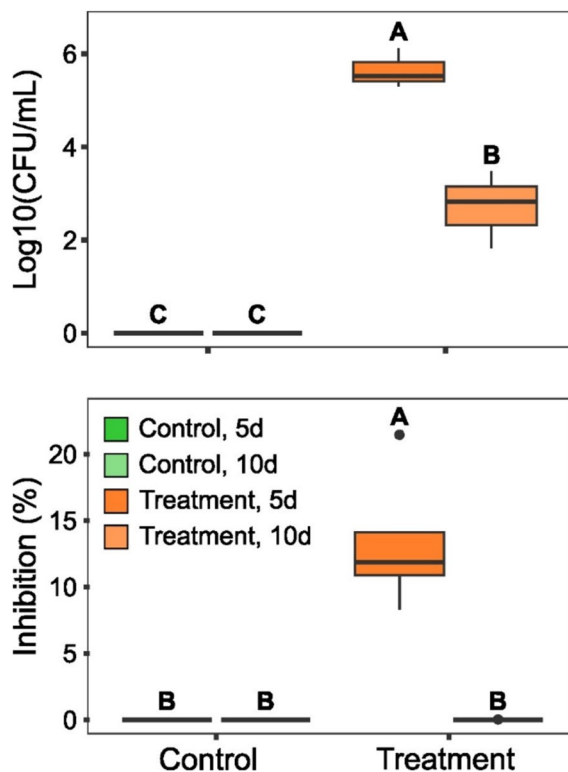


Fig. 1 The effect of KT11 isolate application on the plant sap inhibitory activity against *S. melliferum* *in vitro*. KT11 cells were applied to melon plants (treatment) by foliar spray. The KT11 viable counts (**A**) and inhibitory activity (**B**) were tested 5- and 10- days post-treatment compared to non-inoculated plants (control). Nonparametric ART-anova compared effects of treatment, time, and their interaction. Different letters indicate significant differences ($P < 0.05$)

of fluids found in plant transport tissues: phloem and xylem (Serra-Soriano et al., 2015). The sap of KT11-treated plants contained 10^5 and 10^3 viable *F. defence* cells per milliliter at five- and ten-days post-application, respectively (Fig. 1A). This finding reinforces the fact that KT11 is an endophytic bacterium capable of inhabiting the vascular systems of various plants (Iasur-Kruh et al., 2018; Lidor et al., 2018) while its ability to survive for a long period in the plant environment is limited (Naor et al., 2019). Five days post-application, the sap of KT11-treated plants retained the capability to inhibit *S. melliferum* *in vitro*, while both the sap of control plants and the sap obtained from treated plants ten days post-application did not display any inhibitory effect. Nevertheless, while in the artificial environment (S medium), KT11 cells secreted secondary metabolites, which

caused a high inhibition level (75% - Table 1), under the plant sap environment, the inhibition level was relatively low (13%) but still significant ($P < 0.01$) compared to sap of untreated plants (Fig. 1B).

The differences in inhibition levels between natural medium (sap) and artificial medium (S-growth medium) can be explained by bacterial concentration within the system: The concentration of KT11 in the artificial substrate reached $\sim 10^7$ cells/mL (Naama-Amar et al., 2022), whereas in the natural substrate, concentrations were $\sim 10^5$ and $\sim 10^3$ cells/mL five- and ten-days post-application, respectively. Concurrently, the inhibition levels exhibited a decreasing trend over this time period. This observation suggests a potential direct relationship between the concentration of the bacterial population and the secretion of inhibitory metabolites. The lower counts of KT11 cells in the plant sap could explain the reduced inhibition levels observed in this natural environment compared to the artificial substrate system. Such a relationship between bacterial density and inhibitory metabolite production has also been documented in other bacterial species (Abbasiliasi et al., 2017; Oliveira et al., 2017).

The effect of KT11 on bacterial endophytic community composition

The plant microbiome represents a diverse array of microorganisms interacting with and occupying various niches associated with the host plant (Müller et al., 2016). Introducing an exogenous endophyte like *F. defendens* can induce changes in the composition of the native endophytic bacterial communities of the host plant. For example, certain bacterial populations may become more dominant, while others may decline or even disappear, altering the relative abundances of different endophytic species (Ou et al., 2022; Yurgel et al., 2022). Therefore, the bacteriome of control and treated melon plants (5- and 10-days post-spraying with KT11 isolate) was examined by NGS. β -diversity analysis examined whether the KT11 spraying treatment, the sampling time, and the interaction between these two factors affected the composition of the microbial populations in the different samples. According to the results of the PERMANOVA (Permutational Multivariate Analysis of Variance) statistical test, both the KT11 spraying treatment, and the sampling time significantly

affected the composition of the microbial communities ($P < 0.05$); As a result, certain bacteria, primarily Gammaproteobacteria class, which includes diverse groups with different ecological roles, became more dominant within the system (data not shown). Notably, Gammaproteobacteria and Betaproteobacteria are known to constitute a significant portion of the melon microbiome (Glassner et al., 2015; Saminathan et al., 2018).

Figure 2A illustrates the non-metric multidimensional scaling (NMDS) analysis of bacterial community composition in melon plants treated with the KT11 isolate compared to untreated control plants. The bacterial populations that were significantly affected by KT11 presence are shown in Fig. 2B. As anticipated, *F. defendens* was detected in treated melon plants but absent from untreated control plants. Interestingly, while the absolute concentration of *F. defendens* in plants decreased from five to ten days post-application (Fig. 1), its relative abundance within the endophytic bacterial community increased over this timeframe. A potential explanation is that

the total population of endophytic bacteria declined in the plants, meaning that although *F. defendens* cell numbers dropped, its successful establishment and persistence allowed it to become more prevalent within the diminished endophytic community over time. Indeed, Hardoim et al., 2008 mentioned that endophyte populations tend to be higher in young, developing tissues than in mature plants.

Notably, not only the introduced KT11 isolate was detected in treated melons, but the genus *Herbaspirillum* was also exclusively found in these plants. *Herbaspirillum* was absent from control plants but became the dominant genus in treated plants 5 days after spraying, though its abundance declined 10 days post-treatment. Thus, the relative abundance of this genus exhibited a time-dependent response to the KT11 treatment. *Herbaspirillum* species are known to enhance plant growth and productivity through mechanisms like nitrogen fixation (Alves et al., 2015). Hence, introducing KT11 to melons may foster cooperative interactions with growth-promoting endophytic bacteria in these plants. Moreover, the

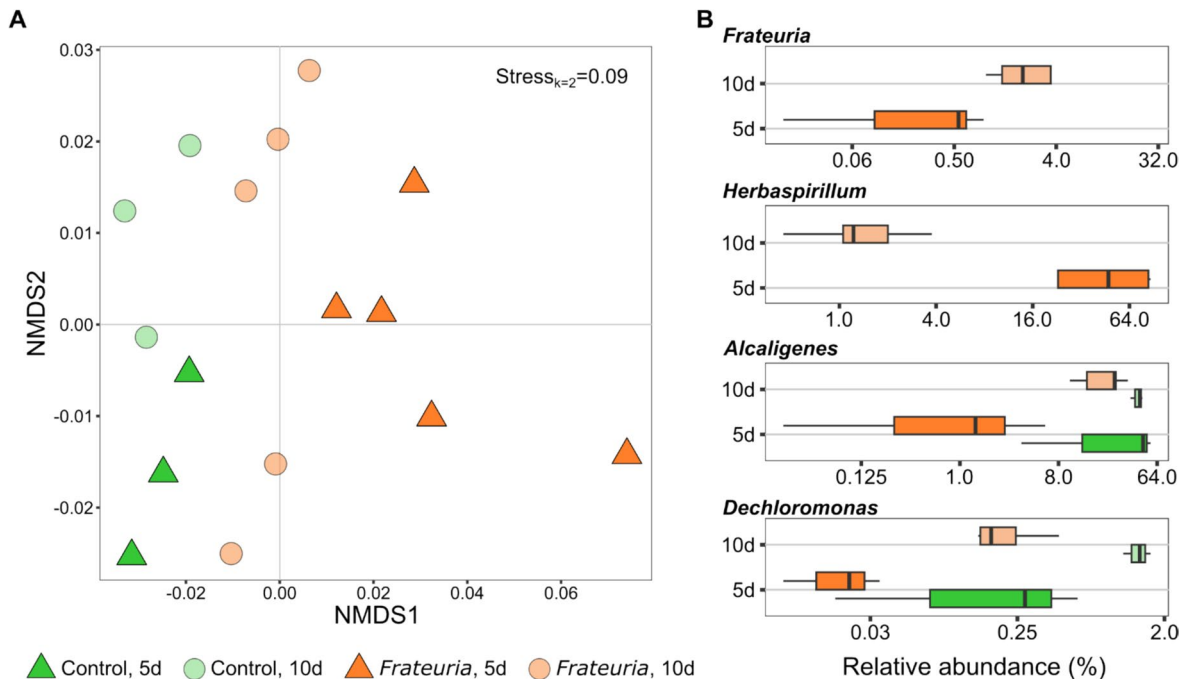


Fig. 2 The effect of KT11 application on the endophytic bacterial community in melon plants. The composition of endophytic communities was determined for melon plants five and ten days after foliar application of the KT11 isolate: **A** Non-metric multidimensional scaling (NMDS) analysis based on a

Bray-Curtis dissimilarity matrix between samples. **B** Selected genera for which relative abundance was significantly affected by treatment ($P < 0.05$, LDA effect size > 2) based on LEfSe analysis. Shapes and colors represent treatment and time points in both panels

introduction of exogenous KT11 led to reductions in certain native bacterial species such as *Alcaligenes* and *Dechloromonas*. *Dechloromonas* displayed a consistent reduction in the relative abundance in both 5 and 10-day time points in treated plants compared to control. While the relative abundance of *Alcaligenes* five days post-application is lower in treated plants compared to control, its level is increased and is closer to the control group after ten days, suggesting a re-establishment of equilibrium. This “return to equilibrium” trend is also reflected in the NMDS plot (Fig. 2A), which shows the variation between treated and untreated populations was greater five days than ten days post-treatment, though not statistically significant. Further investigation at additional time points is needed to determine if a new equilibrium state will be established or if the divergence between treated and untreated bacterial communities will diminish over time.

The current research explores the endophyte-plant relationship from an ecological perspective, assessing the endophyte *F. defendens* (KT11 isolate) impact on melon plants as hosts. The results suggest that this exogenous endophyte influences healthy melon plants in various ways. This includes alterations of the biochemical composition of the plant sap as well as changes in the structure and dynamics of the plant native microbiome. The significant effects of both the treatment and the sampling time on β -diversity highlight the importance of considering both spatial (treatment) and temporal (time) factors when studying the dynamics of microbial communities in response to introducing a biocontrol agent like *F. defendens*.

The present study demonstrates that when the bacterial biocontrol agent *F. defendens* (KT11 isolate) is applied to healthy melon plants, its effect is not visually apparent, but its influence extends beyond what is visible. While it does not cause observable changes in plant morphology (Table S2), KT11 significantly affects the plant's sap components, particularly the inhibitory properties of the sap, and alters the composition of the native endophytic community. These findings support the hypothesis proposed by Yurgel et al. (2022) that the initial introduction of exogenous endophytes can modify the structure and composition of a plant's microbiome. Consequently, these introduced bacteria may foster enhanced cooperation among plant growth-promoting endophytes (such as *Herbaspirillum*), leading to potentially long-term

beneficial effects for the host plant that are not immediately evident.

Funding Open access funding provided by Braude College of Engineering.

Declaration

Conflict of interest The authors declare no conflict of interest.

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