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ORIGINAL RESEARCH

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Metabolic changes in tomato plants caused by psychrotolerant Antarctic endophytic bacteria might be implicated in cold stress mitigation

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Abstract

Climate change is responsible for mild winters and warm springs that can induce premature plant development, increasing the risk of exposure to cold stress with a severe reduction in plant growth. Tomato plants are sensitive to cold stress and beneficial microorganisms can increase their tolerance. However, scarce information is available on mechanisms stimulated by bacterial endophytes in tomato plants against cold stress. This study aimed to clarify metabolic changes stimulated by psychrotolerant endophytic bacteria in tomato plants exposed to cold stress and annotate compounds possibly associated with cold stress mitigation. Tomato seeds were inoculated with two bacterial endophytes isolated from Antarctic *Colobanthus quitensis* plants (*Ewingella* sp. S1.OA.A_B6 and *Pseudomonas* sp. S2.OTC.A_B10) or with *Paraburkholderia phytofirmans* PsJN, while mock-inoculated seeds were used as control. The metabolic composition of tomato plants was analyzed immediately after cold stress exposure (4°C for seven days) or after two and four days of recovery at 25°C. Under cold stress, the content of malondialdehyde, phenylalanine, ferulic acid, and p-coumaric acid was lower in bacterium-inoculated compared to mock-inoculated plants, indicating a reduction of lipid peroxidation and the stimulation of phenolic compound metabolism. The content of two phenolic compounds, five putative phenylalanine-derived dipeptides, and three further phenylalanine-derived compounds was higher in bacterium-inoculated compared to mock-inoculated samples under cold stress. Thus, psychrotolerant endophytic bacteria can reprogram polyphenol metabolism and stimulate the accumulation of secondary metabolites, like 4-hydroxybenzoic and salicylic acid, which are presumably involved in cold stress mitigation, and phenylalanine-derived dipeptides possibly involved in plant stress responses.

1 | INTRODUCTION

Cold stress condition, which can be distinguished in chilling (0–15°C) and freezing (below 0°C), is one of the most common abiotic factors that can limit plant cultivation, productivity, and survival worldwide (Karami-Moalem et al. 2018). Climate change is responsible for global warming, extreme weather events, and cold-air outbreaks, which can intensify the frequency and impacts of cold stress on plants (Gusain et al. 2023). In particular, global warming is causing mild winters and warm or early springs that can induce premature development in several crops, such as tomato (Ventrella et al. 2012), resulting in the damage of vulnerable plant tissues exposed to subsequent cold stress

(Gu et al. 2008; Zohner et al. 2020). Cold stress causes a phase transition of cellular membranes from liquid-crystalline to solid gel structure (membrane rigidification), increasing membrane permeability and electrolyte leakage (Kim et al. 2002). Thus, cold stress negatively affects membrane-associated processes (e.g., photosynthesis and respiration), increasing reactive oxygen species (ROS) content (Theocharis et al. 2012b). When cell membranes are injured, malondialdehyde (MDA) has been reported to be accumulated as a by-product of lipid peroxidation (Uzal 2022). As a consequence, MDA can be used as a marker of cold stress damage in plant species, such as tomato (Uzal 2022). Moreover, plants exposed to cold stress show severe phenotypic symptoms, such as reduced leaf expansion, wilting, leaf chlorosis, and cell necrosis, negatively impacting plant growth (Yadav 2010). Plants try to react to cold exposure by the activation of acclimation processes, which include transcriptional, post-transcriptional, and metabolic (e.g., accumulation of proline, sugars, and other cryoprotectants) mechanisms (Theocharis et al. 2012b; Ding et al. 2019). For example, cold acclimation of tomato plants is known to include the upregulation of cold-related genes, the activation of antioxidant systems, and the accumulation of compatible solutes and anthocyanins (Liu et al. 2012; Barrero-Gil et al. 2016). Likewise, cold stress has been reported to increase the content of phenolic compounds, antioxidant molecules (Samadi et al. 2020; Alhaithloul et al. 2021), and abscisic acid (Heidari et al. 2021) in tomato plants.

Tomato is one of the most economically valuable crops worldwide, but most of the commercially grown cultivars are sensitive to temperatures below 15°C (Van Der Ploeg and Heuvelink 2005; Subramanian et al. 2016). Thus, tomato plants are frequently grown under greenhouses or plastic-covered tunnels in temperate regions to prevent chilling stress (Van Der Ploeg and Heuvelink 2005; Subramanian et al. 2016). Additional strategies can be applied to mitigate severe cold stress on crops, such as overhead irrigation, wind machines, and heating systems (Drepper et al. 2022). However, these methods are expensive and require a large use of natural resources, indicating that alternative approaches for cold stress mitigation are needed to promote sustainable agriculture. For example, treatments with exogenous compounds (e.g., abscisic acid, glutathione, glycine betaine, melatonin, mineral nutrients, potassium nitrate, proline, and salicylic acid) were proposed to improve crop tolerance to cold stress (Baier et al. 2019; Román-Figueroa et al. 2021; Khalid et al. 2022). In particular, treatments with salicylic acid (or methyl salicylate) (Senaratna et al. 2000; Miura and Tada 2014; Orabi et al. 2015; Ding et al. 2016; Meena et al. 2018), ascorbic acid (Elkelish et al. 2020), abscisic acid, polyamines (Kim et al. 2002; Diao et al. 2017), methyl jasmonate (Ding et al. 2022), melatonin (Ding et al. 2017), and proline (Uzal 2022) stimulated cold tolerance in tomato plants. Moreover, beneficial microorganisms in association with tomato plants have been demonstrated to increase tolerance to cold stress, such as *Paraburkholderia phytofirmans* PsJN (*Paraburkholderia* sp.) (Perazzolli et al. 2022), *Flavobacterium* sp. OB146 and *Pseudomonas* sp. OB155, OS261, and OS211 (Subramanian et al. 2015, 2016), *Trichoderma asperellum* HK703 (Cornejo-Ríos et al. 2021), *T. harzianum* AK20G (Ghorbanpour et al. 2018), *Streptomyces* sp. TOR3209 (Ma et al. 2022), *Mrakia* sp. Mr1, *Naganishia* sp. Ng1, *Pseudomonas* sp. Ps1, *Rhodotorula* sp. Rh2 and Rh3 (Tapia-Vázquez et al. 2020), *Pseudomonas* sp. TPs-04 (Chen et al. 2014) and bacterial consortia, such as *Bacillus* sp. and *Serratia* sp. isolates (Wang et al. 2016) or *Curtobacterium* sp., *Frondihabitans* sp., and *Pseudomonas* sp. isolates (Vega-Celedón et al. 2021).

Complex microbial communities are known to be associated with plants in cold environments and they can contribute to plant growth under cold stress (Marian et al. 2022). For example, *Duganella*, *Ewingella*, *Hafnia*, *Janthinobacterium*, *Pedobacter*, *Pseudomonas*, and *Rahnella* genera were the most abundant psychrotolerant endophytic bacteria found in Antarctic *Colobanthus quitensis*, and their possible contribution to plant tolerance under cold conditions was hypothesized (Perazzolli et al. 2022). In particular, two psychrotolerant bacterial isolates, named *Ewingella* sp. S1.OA.A_B6 (*Ewingella* sp.) and *Pseudomonas* sp. S2.OTC.A_B10 (*Pseudomonas* sp.), showed growth promotion activity in tomato seedlings at low temperatures (Perazzolli et al. 2022). However, no information is available on the possible mechanisms responsible for cold stress mitigation. Some plant-associated microorganisms can activate acclimation processes and exert positive effects on plant performance at low temperatures by triggering hormone signaling, antioxidant responses, and osmolyte accumulation to improve nutrient acquisition, photosynthesis efficiency, and biomass production (Acuña-Rodríguez et al. 2020). Beneficial effects of bacterial inoculation included the modulation of cold-related genes and the reduction in cellular damages (e.g., MDA content) under cold stress (Subramanian et al. 2015, 2016; Wang et al. 2016; Ma et al. 2022). However, no information is available on metabolic changes activated in bacterium-inoculated tomato plants under cold stress. The aim of this study was to clarify metabolic processes stimulated by psychrotolerant endophytic bacteria in tomato plants exposed to cold stress and to annotate compounds possibly associated with cold stress mitigation.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates and inoculum preparation

The psychrotolerant bacteria *Ewingella* sp. S1.OA.A_B6 (*Ewingella* sp.; NCBI accession number MZ089444; previously annotated as *Hafnia* sp. S1.OA.A_B6) and *Pseudomonas* sp. S2.OTC.A_B10 (*Pseudomonas* sp.; NCBI accession number MZ089387) were previously isolated from surface-sterilized leaves of *C. quitensis* and showed plant growth promotion activity on tomato seedlings at low temperatures (Perazzolli et al. 2022). As a control, *Paraburkholderia phytofirmans* PsJN (*Paraburkholderia* sp.) was used due to its ability to improve plant tolerance to cold stress (Ait Barka et al. 2006; Fernandez et al. 2012a; Fernandez et al. 2012b; Theocharis et al. 2012) and to promote tomato growth at low temperatures (Perazzolli et al. 2022). Bacterial isolates were stored in 40% glycerol at −80°C and grown on solid Antarctic bacterial medium (ABM; 5 g L^{-1} peptone, 2 g L^{-1} yeast extract, and 15 g L^{-1} agar; Oxoid Ltd.) at 25 ± 1°C.

To prepare the bacterial suspension for seed inoculation, each isolate was grown overnight (18 h) in liquid ABM (5 g L^{-1} peptone

and 2 g L^{-1} yeast extract; Oxoid Ltd.) at 25 ± 1°C under orbital shaking at 200 rpm. Bacterial cells were collected by centrifugation (3,500 × *g* for 10 min) and washed three times with sterile 10 mM $MgSO_4$. The bacterial suspension was then adjusted to 1.0×10^9 colony forming units (CFU) per unit of volume (CFU mL^{-1}) based on the conversion of optical density (OD) at 600 nm (OD_{600}) previously optimized for each isolate ($OD_{600} = 0.1$ corresponded to 2.3×10^8 CFU mL^{-1} *Paraburkholderia* sp., 2.0×10^8 CFU mL^{-1} *Ewingella* sp., and 3.5×10^7 CFU mL^{-1} *Pseudomonas* sp.) (Perazzolli et al. 2022) and assessed with an Ultrospec 3100 spectrophotometer (GE Healthcare).

2.2 | Assessment of indoleacetic acid production by bacterial isolates

The production of indoleacetic acid by bacterial isolates was assessed as previously described (Gang et al. 2019), with slight modifications. Briefly, each bacterial isolate was grown in liquid ABM supplemented or not supplemented with 0.5 mg mL^{-1} tryptophan (Acros Organics BV) by incubation at 25 ± 1°C or 4 ± 1°C under orbital shaking at 200 rpm for 48 h. The OD_{600} of each bacterial suspension was assessed at the end of the incubation period using an Ultrospec 3100 pro spectrophotometer (GE HealthCare) to calculate the number of CFU mL^{-1}. Cells were collected by centrifugation (3,500 × *g* for 10 min) and 1 mL of the supernatant was mixed with 1 mL of Salkowski reagent (10 mM $FeCl_3$ in 35% perchloric acid). The mixture was incubated at room temperature for 30 min in the dark and the OD at 536 nm was assessed to quantify the indoleacetic acid content, using a calibration curve of 0, 5, 10, 20, 50, and 100 μg mL^{-1} indoleacetic acid dissolved in liquid ABM. The content of indoleacetic acid was normalized according to the CFU number of the respective bacterial suspension. Three replicates (bacterial cultures) were assessed for each isolate, culture medium, and incubation temperature, and the experiment was carried out twice.

2.3 | Tomato seed inoculation, growth conditions, and cold stress treatment

Seeds of *Solanum lycopersicum* L. cultivar Moneymaker (Justseed Ltd.) were surface disinfected as previously reported (Perazzolli et al. 2022). Briefly, seeds were treated with 70% ethanol for 1 min, 2% sodium hypochlorite for 5 min, and 70% ethanol for 1 min, followed by three washes with sterile distilled water (3 min each) in a 15-mL tube with moderate shaking. Surface-disinfected seeds (150 seeds for each inoculum condition) were treated with 5 mL of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 5 mL of the bacterial suspension (bacterium-inoculated) of the respective isolate (1.0×10^9 CFU mL^{-1} of *Paraburkholderia* sp., *Ewingella* sp., or *Pseudomonas* sp.) by overnight (18 h) incubation at 25 ± 1°C in a 15-mL tube under orbital shaking in the dark at 70 rpm. Seeds of each inoculation were transferred into a 100 cm^2-square dish (150 seeds per dish; Sarstedt AG) containing 10 g L^{-1} water agar (Oxoid Ltd.) and they were incubated for two days in a growth chamber (Kälte Klima Röhler) at 25 ± 1°C with a photoperiod of 16 h light and 8 h dark to allow seed germination. Germinated seeds with the same root length (about 2 mm) were selected and each germinated seed was transferred in a glass tube for plant tissue culture (30 mm diameter and 150 mm height; Artiglass) containing 15 mL of half-strength Hoagland medium with 6 g L^{-1} agar (Oxoid Ltd.). Plants were grown for 42 days in the growth chamber (25 ± 1°C with a photoperiod of 16 h light and 8 h dark) and exposed to cold stress at 4 ± 1°C in the dark for seven days (cold-stressed plants, Figure S1). This incubation period was chosen to analyze the tomato response to cold stress at 4 ± 1°C, as previously described (Wang et al. 2016). Plants were transferred back to the growth chamber to allow the recovery from stress (Wang et al. 2016; Yu et al. 2016). Mock-inoculated and bacterium-inoculated samples were collected immediately after cold stress exposure (0 recovery), and after two (2 recovery) or four (4 recovery) days of recovery in the growth chamber at 8 a.m., before the light was switched on (Figure S1). As control, mock-inoculated and bacterium-inoculated plants were incubated in the growth chamber for 49 days before sample collection (non-stressed plants; Figure S1). For each inoculum condition (mock-, *Paraburkholderia*-, *Ewingella*-, or *Pseudomonas*-inoculated) and temperature regime (non-stressed and cold-stressed at 0 recovery, 2 recovery, and 4 recovery), the shoot (stem and leaves) of tomato plants was collected and, for each replicate, a pool of five plants was immediately frozen in liquid nitrogen. Tomato shoots were ground using a mixer-mill disruptor (MM 400, Retsch) with refrigerated stainless jars at 25 Hz for 30 sec and the resulting plant powder was stored at −80°C until further analysis. Since the incubation in the dark could partially affect plant response to cold stress and the biosynthesis of secondary metabolites (Kula-Maximenko et al. 2021), pairwise comparisons between bacterium-inoculated and mock-inoculated samples were carried out for each temperature regime to detect the effects of bacterial inoculation in plants incubated in the same light/dark condition. Roots of each plant were plated on solid ABM to verify the presence and absence of bacterial colonization in bacterium-inoculated and mock-inoculated samples, respectively. Five replicates (a pool of five plants each) were analyzed for each inoculum condition and temperature regime.

2.4 | Bacterial re-isolation from tomato plants

After 14 and 49 days of incubation in the growth chamber, mock-inoculated and bacterium-inoculated samples were collected to verify endophytic bacterial colonization. The shoot (stem and leaves) of each plant was weighted and surface-disinfected in a 50-mL tube with 70% ethanol for 1 min, 2% sodium hypochlorite for 1 min, and 70% ethanol for 1 minute, as previously described (Galambos et al. 2020). Each replicate (plant) was washed three times with sterile distilled water (3 min each), dried with a sterile filter paper, and ground in the presence of 500 μL sterile 10 mM $MgSO_4$ using a mixer-mill disruptor (MM 400, Retsch) with a sterile stainless jar at 25 Hz for 45 sec. Each suspension was serially diluted in sterile 10 mM $MgSO_4$ and plated on

solid ABM in triplicate. As control of plant surface disinfection, the last washing solution (20 mL) was centrifuged (3,500 *g* for 10 min), the supernatant (19.5 mL) was discarded and aliquots (20 µL) of the remaining solution (500 µL) were plated to confirm the absence of bacterial growth. CFU values of endophytic bacteria were calculated per gram of fresh weight (CFU g^{-1}) after three days of incubation at 25 ± 1°C. Three replicates (plants) were analyzed for each inoculum condition and time point.

2.5 | Malondialdehyde quantification

Malondialdehyde (MDA) content was assessed in tomato samples with the colorimetric assay as previously reported (Theocharis et al. 2012a) with slight modifications. Briefly, an aliquot of each tomato sample (50 ± 2 mg of ground tomato shoots) was weighed into a 2-mL tube and 0.5 mL of 0.1% (w v^{-1}) trichloroacetic acid (4°C) was added. After vigorous shaking, each sample was centrifuged at 12,000 × *g* for 10 min at 4°C, 0.5 mL of each supernatant was collected in a new 2-mL tube and mixed with 0.5 mL of 20% (w v^{-1}) trichloroacetic acid containing 0.5% (w v^{-1}) 2-thiobarbituric acid. The mixture was incubated at 95°C for 30 min, cooled in ice, and centrifuged at 10,000 × *g* for 10 min at 4°C before assessing the OD at 532 nm and 600 nm. The nonspecific absorbance at 600 nm was subtracted from the maximum absorbance at 532 nm, the MDA content in each sample was calculated according to the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as µmol of MDA per gram of fresh weight. Five replicates (a pool of five plants each) were analyzed for each inoculum condition and temperature regime.

2.6 | Targeted metabolomic analysis of phenolic compounds by high-performance liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis

An aliquot of each tomato sample (50 ± 2 mg of ground tomato shoots) was weighed into a 1.5-mL tube and extracted with 1 mL of cool (4°C) extraction solvent [methanol:water (3:1; v v^{-1}; water purified with an ELGA Purelab Ultra-AN-MK2 system, Veolia Water) containing 0.1% (v v^{-1}) formic acid (Sigma-Aldrich)] using an ultrasonic bath (Branson 2210, Bransonic) for 15 min. Samples were centrifuged at 18,000 × *g* for 10 min at 4°C, the supernatant (300 µL) was collected in a new 1.5-ml tube at 4°C, and 150 µL of 0.1% (v v^{-1}) formic acid in water were added to each sample to achieve 50% content of organic solvent for subsequent LC-HRMS analysis [methanol:ELGA water (1:1; v v^{-1}) containing 0.1% formic acid; injection solvent]. Samples were centrifuged at 18,000 × *g* for 10 min at 4°C, and the supernatant (150 µL) was transferred into a high-performance liquid chromatography (HPLC) vial (Bruckner Analysentechnik). For quality control (QC) assessment (Bueschl et al. 2014), equal amounts (10 µL) of each tomato plant extract were mixed to yield an aggregate sample. At least four replicates (a pool of five plants each) were subjected to high-performance liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis for each inoculum condition and temperature regime in a randomized complete block design, and an aggregate sample was analyzed every twelve samples using a VH-A10 auto-sampler at 10°C (Vanquish, Thermo Fisher Scientific).

For targeted metabolomic analysis, the following chemical standards of phenolic compounds were analyzed: 4-hydroxybenzoic acid (not specified purity, Merck), catechin (>99% pure, Extrasynthese), catechol (>99% pure, Sigma-Aldrich, Merck), chlorogenic acid (>95% pure, Sigma-Aldrich, Merck), epicatechin (>98% pure, Sigma-Aldrich, Merck), ferulic acid (>99% pure, Fluka, Merck), gallic acid (>99% pure, Extrasynthese), p-coumaric acid (not specified purity, Sigma-Aldrich, Merck), phenylalanine (>99% pure, ROTH), resveratrol (>99% pure, Sigma-Aldrich, Merck), rosmarinic acid (>98%, Sigma-Aldrich, Merck), rutin (analytical standard, Fluka, Merck), salicylic acid (>99% pure, Sigma-Aldrich, Merck), syringic acid (>97% pure, Fluka, Merck), and vanillic acid (>97% pure, Fluka, Merck). These phenolic compounds were selected from the literature according to their potential involvement in tomato response to cold stress (Alhaithloul et al. 2021). Each chemical standard (50 mg) was weighted using an analytical balance (M500P, Sartorius), dissolved in 10 mL methanol:water (3:1; v v^{-1}), diluted to 50 mg L^{-1} in the injection solvent, and stored at −20°C. Equal amounts of each chemical standard were mixed in the injection solvent and this mixed standard stock solution (2 mg L^{-1} of each chemical standard) was used to establish a calibration series of 0, 2, 5, 10, 25, 50, 100, 200, 500, 1,000 and 2,000 µg L^{-1} for each chemical standard in duplicate.

A Vanquish Horizon HPLC system (Thermo Fisher Scientific) coupled with a QExactive HF Orbitrap (Thermo Fisher Scientific) and equipped with a heated electrospray ionization (ESI) source was used for the LC-HRMS analysis. A reversed-phase XBridge C_{18} analytical column (150 × 2.1 mm of inner diameter, 3.5 µm particle size; Waters), preceded by a C_{18} security cartridge (4 × 3 mm inner diameter; Phenomenex), was used for chromatographic separation with a constant flow rate of 250 µL min^{-1} at 25°C, and an injection volume of 4 µL. A linear elution gradient was achieved using 0.1% (v v^{-1}) formic acid (eluent A) and 100% (v v^{-1}) methanol containing 0.1% (v v^{-1}) formic acid (eluent B). The initial mobile phase (10% eluent B) was held constant for 2 min, followed by a linear gradient to 100% eluent B within 30 min. After a hold time of 5 min with 100% eluent B at the end of each run, the column was re-equilibrated for 8 min at 10% eluent B, and the needle was washed with the injection solvent before injection of a new sample. The ESI interface was operated in polarity switching mode with the following settings, sheath gas: 55 arbitrary units, auxiliary gas: 5 arbitrary units, capillary voltage: 3,5 kV in positive ESI mode and 3 kV in negative ESI mode, capillary temperature: 320°C. For measurements in the full scan mode, the automatic gain control was set to a target value of 3×10^6 and a maximum injection time of 200 ms. Mass spectra were recorded in profile mode with a scan range *m/z* from 100 to 1,000 and a resolving power setting of 120,000 full width at half maximum at *m/z* 200 (Doppler et al. 2019a).

Raw data were acquired with the Xcalibur software version 4.2.28.14 (Thermo Fisher Scientific) and the abundance of each

feature was calculated as the integrated peak area of the extracted ion chromatogram (peak area). Peak detection and peak inspection were performed using the Quanl Browser function of the Xcalibur software. Missing values were replaced by the lowest value of the respective feature in the data set ±40% random variation to keep the variance of the feature as previously reported (Warth et al. 2015). The linear calibration curve was constructed for each chemical standard using integrated peak areas as derived by the Quan Browser function of the Xcalibur (Thermo Fisher Scientific), and the content of phenolic compounds in each sample was expressed as µg per g of fresh weight (µg g^{-1} FW).

2.7 | Annotation of phenylalanine-derived compounds

In order to annotate phenylalanine-derived compounds in tomato plant extracts, wheat extracts from a previous isotope-assisted study with $^{13}C_9$ phenylalanine (99% isotopic purity; Eurisotop) as a metabolic tracer were provided by Doppler and colleagues (Doppler et al. 2019a) and used as reference samples in the LC-HRMS analysis. Extracts of $^{13}C_9$ phenylalanine-treated wheat samples were analyzed within the same sequence as the tomato plant extracts and used to automatically search for $^{13}C_9$ phenylalanine-derived features. To this end, chromatograms of wheat extracts were processed with the TracExtract module of MetExtract II software (Bueschl et al. 2017), according to the parameter settings reported in Table S1, to find co-eluting pairs of unlabeled and labeled features in wheat extracts. Phenylalanine-derived features were detected in wheat extracts, and the number of phenylalanine-derived carbon atoms was determined for each compound based on the difference between the ^{13}C-labeled mass and the native mass. Features possibly originated from the same compound were clustered into the same feature group by a pairwise Pearson correlation analysis ($R^2 \geq 0.85$), which was used to cluster all features per metabolite into feature groups (Bueschl et al. 2017). Feature annotation was based on predicted ion species, *m/z* values, and the number of phenylalanine-derived carbon atoms using the MetExtract II software (Bueschl et al. 2017), and matching against the TomatoCyc database (https://plantcyc-ftp.storage.googleapis.com/pmn/Pathways/Data_dumps/PMN15_January2021/compounds/tomato_compounds.20210325.txt; January 2021; 12,000 entries), two in-house built databases of wheat metabolites (WheatA with 1,100 entries and WheatD with 1,200 entries) (Doppler et al. 2019a), the Chemical Entities of Biological Interest database (136,000 entries) (Degtyarenko et al. 2008), and the Chemical Component Dictionary database (467,000 entries) (Westbrook et al. 2015).

In order to find phenylalanine-derived features in tomato plant extracts, chromatographic peaks detected in wheat samples were searched in tomato chromatograms using the reintegration and bracketing function of MetExtract II with retention time and *m/z* values of phenylalanine-derived compounds of wheat samples as reference parameters (Bueschl et al. 2017). The most abundant feature (average of the integrated peak area in tomato plant extracts) of each feature group was used to represent the respective compound (Doppler et al. 2019b) and its content in tomato plant extracts was expressed as peak area divided by the plant fresh weight in micrograms (peak area µg $^{-1}$ FW). After peak integration in chromatograms of tomato samples, phenylalanine-derived dipeptides and other phenylalanine-derived compounds were selected (Doppler et al. 2019a). Putative annotations were manually curated to verify the presence of phenylalanine-derived moieties in the molecular structures of annotated features found by MetExtract II and one, or more, of the most probable annotations were selected for each feature.

2.8 | Statistical analysis

Statistical analyses were carried out using Past 4.03 software (Hammer et al. 2001). The Kruskal-Wallis test and Mann–Whitney test were used to detect significant differences ($P \leq 0.05$) among treatments in multiple and pairwise comparisons, respectively. In particular, the Mann–Whitney test was used to detect significant differences ($P \leq 0.05$) between bacterium-inoculated and mock-inoculated samples for each temperature regime (non-stressed and cold-stressed at 0 recovery, 2 recovery, and 4 recovery).

3 | RESULTS

3.1 | *Ewingella* sp., *Paraburkholderia* sp., and *Pseudomonas* sp. colonize tomato plants and limit lipid peroxidation under cold stress

To test plant growth promotion traits under cold conditions, indoleacetic acid production by psychrotolerant endophytic bacteria was evaluated *in vitro*. Indoleacetic acid was produced by *Ewingella* sp., but not *Paraburkholderia* sp. and *Pseudomonas* sp., when grown in the liquid medium supplemented with tryptophan, and its content was comparable after incubation at 25°C and 4°C (Figure S2).

To verify the possible effect of psychrotolerant endophytic bacteria in the mitigation of cold stress in tomato plants, seed inoculation was carried out with *Paraburkholderia* sp, *Ewingella* sp., or *Pseudomonas* sp., and the content of a marker of cold stress damage (MDA) was assessed in shoots of non-stressed plants or plants exposed to cold stress (4 ± 1°C for seven days) and collected immediately after stress exposure, or after two and four days of recovery at 25 ± 1°C (Figure S1). *Paraburkholderia* sp., *Ewingella* sp., and *Pseudomonas* sp. were able to endophytically colonize tomato plants and they were re-isolated from surface-sterilized tomato shoots at comparable levels after 14 and 49 days of incubation in the growth chamber (Figure S3). The content of MDA increased in cold-stressed compared to non-stressed plants, while it was lower in *Paraburkholderia*, *Ewingella*, and *Pseudomonas*-inoculated samples compared to mock-inoculated samples after zero, two, and four days of recovery (Figure 1), as possible mitigation of lipid peroxidation in bacterium-inoculated plants.

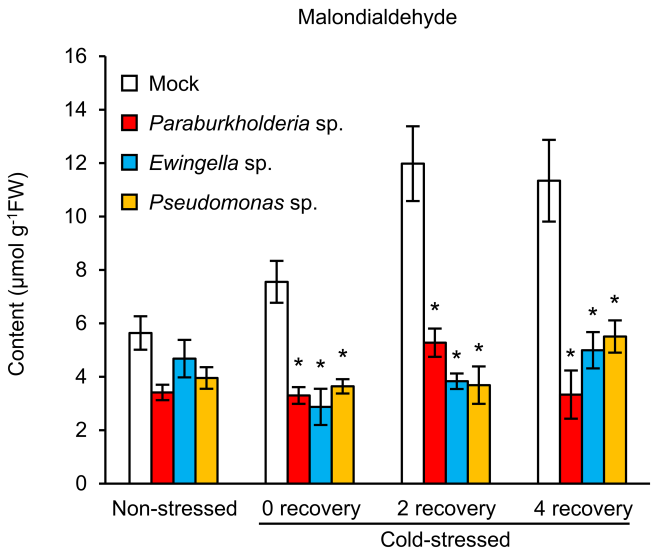

FIGURE 1 Content of malondialdehyde in tomato samples. Tomato seeds were treated with sterile 10 mM $MgSO_4$ (mock-inoculated; white bars) or inoculated with *Paraburkholderia phytofirmans* PsJN (red bars), *Ewingella* sp. S1.OA.A_B6 (blue bars), and *Pseudomonas* sp. S2.OTC.A_B10 (yellow bars). Plants were exposed or not (non-stressed; 25 ± 1°C) to cold stress (cold-stressed; 4 ± 1°C) and collected immediately after cold stress exposure (0 recovery), or after two (2 recovery) and four (4 recovery) days of recovery at 25 ± 1°C. Malondialdehyde content was assessed by a colorimetric assay and expressed as µmol per gram fresh weight (µmol g^{-1} FW). Mean and standard error values of five replicates (a pool of five plants each) are reported for each inoculum condition and temperature regime. Asterisks indicate significant differences in metabolite content between bacterium-inoculated and mock-inoculated plants for each temperature regime, according to the Mann–Whitney test ($P \leq 0.05$).

3.2 | *Ewingella* sp., *Paraburkholderia* sp., and *Pseudomonas* sp. inoculation affect the content of phenolic compounds in tomato plants under cold stress

Targeted metabolomic analysis of phenolic compounds was carried out by LC-HRMS on mock-, *Paraburkholderia*-, *Ewingella*-, or *Pseudomonas*-inoculated shoot samples of non-stressed plants or plants exposed to cold stress and collected immediately after stress exposure, or after two and four days of recovery. Seven phenolic compounds (4-hydroxybenzoic acid, chlorogenic acid, ferulic acid, p-coumaric acid, phenylalanine, rutin, and salicylic acid) were found and quantified in tomato plant extracts by targeted metabolomic analysis, while the other target compounds were not detected, such as catechin, catechol, epicatechin, gallic acid, resveratrol, rosmarinic acid, syringic acid, and vanillic acid (Table S2). In particular, the content of phenylalanine was lower in *Paraburkholderia*-, *Ewingella*-, and *Pseudomonas*-inoculated compared to mock-inoculated samples after two and four days of recovery (Figure 2A). Likewise, the content of ferulic acid was lower in *Ewingella*- and *Pseudomonas*-inoculated compared to mock-inoculated samples after two days of recovery (Figure 2B), and the content of p-coumaric acid was lower in *Paraburkholderia*-inoculated samples after four days of recovery (Figure 2C). Moreover, the content of chlorogenic acid was lower in bacterium-inoculated compared to mock-inoculated samples after two (*Paraburkholderia* inoculation) and four (*Paraburkholderia*, *Ewingella*, and *Pseudomonas* inoculation) days of recovery (Figure 2D), and the content of rutin was lower in *Paraburkholderia*- and *Pseudomonas*-inoculated compared to mock-inoculated samples (Figure 2E). Conversely, the content of salicylic acid was higher in bacterium-inoculated compared to mock-inoculated samples in cold-stressed plants after zero (*Paraburkholderia*, *Ewingella*, and *Pseudomonas* inoculation), two (*Paraburkholderia* inoculation), and four (*Paraburkholderia* inoculation) days of recovery (Figure 2F). Moreover, the content of 4-hydroxybenzoic acid was higher in *Ewingella*- and *Paraburkholderia*-inoculated compared to mock-inoculated samples after zero and two days of recovery, respectively (Figure 2G).

3.3 | *Ewingella* sp., *Paraburkholderia* sp., and *Pseudomonas* sp. inoculation affect the content of additional polyphenolic compounds in tomato plants under cold stress as derived from $^{13}C_9$-phenylalanine tracer analysis

With the $^{13}C_9$-phenylalanine tracer approach, a total of 394 phenylalanine-derived features were found in wheat extracts from a previous isotope-assisted study with $^{13}C_9$ phenylalanine (Doppler et al. 2019a) by LC-HRMS analysis and they were grouped in 144 putative compounds (Table S3). In particular, 63 phenylalanine-derived compounds were found in tomato plant extracts after a manually curated annotation by checking the presence of phenylalanine-derived structure moieties in the annotated features, and one, or more, most probable annotation among the suggested annotations were selected for each feature (Table S4). Among those, five phenylalanine-derived dipeptides (Figure 3) and four further phenylalanine-derived compounds (Figure 4) showed higher content in bacterium-inoculated compared to mock-inoculated samples, while the other 54 phenylalanine-derived compounds were differently affected by inoculation condition and temperature regime (Table S4). For example, *Ewingella* and *Pseudomonas* inoculation reduced the content of four compounds after two days of recovery (putative dihydroxy cinnamoyl amino hydroxybutanoic acid, two putative naringenin derivatives, and a compound annotated as putative vanillic acid glucuronide, coumaroyl glucose, or dihydroxycinnamoyl hexose), and two compounds after four days of recovery (putative feruloyl hexose and putative coumaroyl hexose isomer or glucosyloxy cinnamic acid; Table S4).

In more detail, a compound annotated as putative phenylalanine-phenylalanine was more abundant in bacterium-inoculated compared to mock-inoculated samples in non-stressed plants (*Pseudomonas* inoculation), and cold-stressed plants after zero (*Paraburkholderia*, *Ewingella*, and *Pseudomonas* inoculation) and two (*Paraburkholderia* and *Pseudomonas* inoculation) days of recovery (Figure 3A). The content of putative phenylalanyl-glutamic acid anhydride was higher in

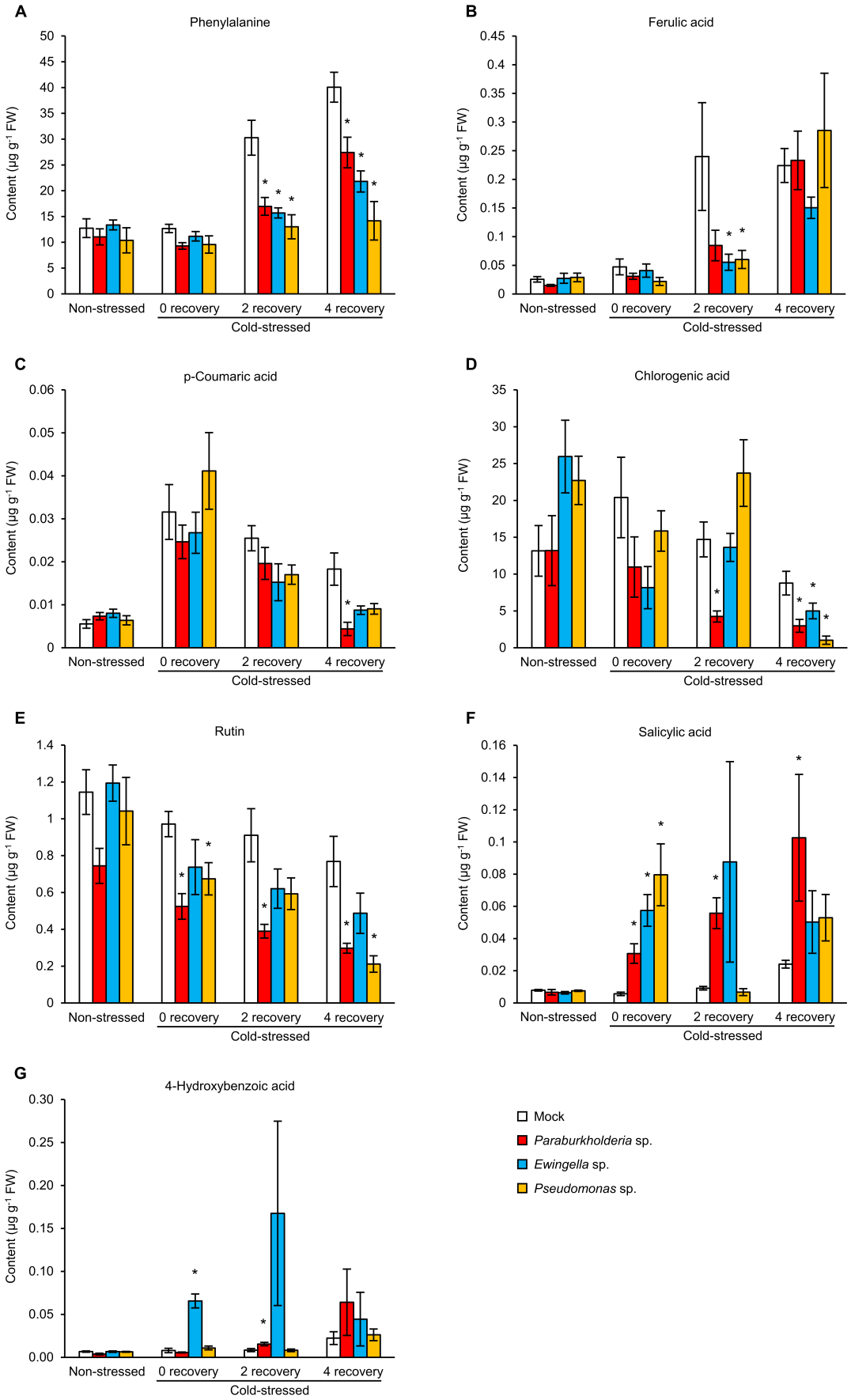

FIGURE 2 Legend on next page.

Pseudomonas-inoculated compared to mock-inoculated samples after two and four days of recovery (Figure 3B), and the content of putative phenylalanine-glycine was higher in *Pseudomonas*- and *Paraburkholderia*-inoculated compared to mock-inoculated samples after zero and two days of recovery, respectively (Figure 3C). A compound annotated as putative phenylalanine-proline was more abundant in bacterium-inoculated compared to mock-inoculated samples (*Pseudomonas* inoculation in non-stressed plants and after zero and four days of recovery, *Paraburkholderia* inoculation after four days of recovery; Figure 3D). Likewise, the content of putative phenylalanine-glutamine was higher in bacterium-inoculated compared to mock-inoculated samples after zero (*Ewingella* and *Pseudomonas* inoculation), two, and four (*Paraburkholderia* inoculation) days of recovery (Figure 3E).

The content of putative vanilloloside was higher in bacterium-inoculated compared to mock-inoculated samples after zero (*Ewingella* and *Pseudomonas* inoculation) and two (*Paraburkholderia*, *Ewingella*, and *Pseudomonas* inoculation) days of recovery (Figure 4A). In addition, a structurally closely related oxidation product, annotated as putative vanilloyl hexose, was more abundant in *Pseudomonas*-inoculated than in mock-inoculated samples in all temperature regimes (Figure 4B). The content of putative sinapate was higher in bacterium-inoculated compared to mock-inoculated samples (*Paraburkholderia* and *Ewingella* inoculation in non-stressed plants and cold-stressed plants after zero days of recovery, *Paraburkholderia* inoculation after four days of recovery; Figure 4C). The content of a compound annotated as putative feruloyl-dihexoside, or an isomer thereof, was higher in *Paraburkholderia*-, *Ewingella*-, and *Pseudomonas*-inoculated compared to mock-inoculated samples in non-stressed plants (Figure 4D).

4 | DISCUSSION

Beneficial bacteria can increase tomato tolerance to cold stress (Chen et al. 2014; Subramanian et al. 2015, 2016; Wang et al. 2016; Acuña-Rodríguez et al. 2020; Vega-Celedón et al. 2021; Ma et al. 2022). In particular, *Ewingella* sp., *Paraburkholderia* sp., and *Pseudomonas* sp. promoted shoot length and fresh weight of tomato seedlings grown at low temperatures (Perazzolli et al. 2022), but no information is available on metabolic changes associated with cold stress mitigation in bacterium-inoculated tomato plants. In agreement with previous findings (Uzal 2022), the content of MDA increased in cold-stressed compared to non-stressed plants, indicating the lipid peroxidation in tomato samples collected after two and four days of recovery in our experiments. Although plant incubation under dark conditions could partially affect the response to cold stress and the biosynthesis of secondary metabolites (Kula-Maximenko et al. 2021), effects of bacterial inoculation were detected in our study by pairwise comparisons between bacterium-inoculated and mock-inoculated samples for each temperature regime, comparing plants incubated in the same light/dark condition. In particular, *Ewingella*, *Paraburkholderia*, and *Pseudomonas* inoculation reduced the content of MDA in cold-stressed plants, indicating the mitigation of cold stress damage in tomato plants by the colonization of these psychrotolerant endophytic bacteria. A lower content of MDA was also previously found in cold-stressed tomato plants inoculated with *Pseudomonas* sp. TPs-04 (Chen et al. 2014), *Streptomyces* sp. TOR3209 (Ma et al. 2022), *Flavobacterium* sp. OB146, *Pseudomonas* sp. OB155, OS261, and OS211 (Subramanian et al. 2015, 2016). Likewise, *Paraburkholderia*-inoculation has previously been shown to reduce MDA content in grapevine plants exposed to cold stress (Theocharis et al. 2012a), indicating that different beneficial bacteria can decrease lipid peroxidation in plants, thereby mitigating cold stress damage.

The content of phenylalanine, ferulic acid, and p-coumaric acid tended to increase in cold-stressed compared to non-stressed tomato plants, and this increase was limited in bacterium-inoculated compared to mock-inoculated samples, suggesting that stress responses were mitigated by bacterial inoculation. It was previously reported that phenylalanine content increases in cold-stressed Tartary buckwheat landraces (Song et al. 2022), while ferulic acid and p-coumaric acid content increases in cold-stressed tomato fruits (Shu et al. 2022) and plants (Alhaithloul et al. 2021), respectively. Moreover, the exogenous application of phenylalanine (Aghdam et al. 2019) and ferulic acid (Shu et al. 2022) promoted cold tolerance in tomato fruits by the modulation of cold stress-related genes, the regulation of antioxidant processes and the activation of enzymes of the secondary metabolism (e.g., phenylalanine ammonia-lyase). In particular, phenylalanine and closely related downstream derivatives, such as ferulic acid and p-coumaric acid, are key precursors in the biosynthesis of secondary metabolites involved in the protection against stresses (Vogt 2010). Thus, endophytic colonization of *Ewingella*, *Paraburkholderia*, and *Pseudomonas* could stimulate the metabolism of ferulic acid, p-coumaric acid, and phenylalanine in tomato plants to synthesize phenolic compounds and phenylalanine-derived compounds possibly implicated in

FIGURE 2 Content of phenolic compounds in tomato samples. Tomato seeds were treated with sterile 10 mM $MgSO_4$ (mock-inoculated; white bars) or inoculated with *Paraburkholderia phytofirmans* PsJN (red bars), *Ewingella* sp. S1.OA.A_B6 (blue bars), and *Pseudomonas* sp. S2.OTC.A_B10 (yellow bars). Plants were exposed or not (non-stressed; 25 ± 1°C) to cold stress (cold-stressed; 4 ± 1°C) and collected immediately after cold stress exposure (0 recovery), or after two (2 recovery) and four (4 recovery) days of recovery at 25 ± 1°C. Plants were exposed to cold stress (4 ± 1°C) and collected before (non-stressed) and immediately after cold stress exposure (0 recovery), or after two (2 recovery) and four (4 recovery) days of recovery. The content of phenylalanine (A), ferulic acid (B), p-coumaric acid (C), chlorogenic acid (D), rutin (E), salicylic acid (F), 4-hydroxybenzoic acid (G), was assessed by high-performance liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis and expressed as μg per g of fresh weight (μg g^{-1} FW). Mean and standard error values of at least four replicates (a pool of five plants each) are reported for each inoculum condition and temperature regime. Asterisks indicate significant differences in metabolite content between bacterium-inoculated and mock-inoculated plants for each temperature regime, according to the Mann–Whitney test ($P \leq 0.05$).

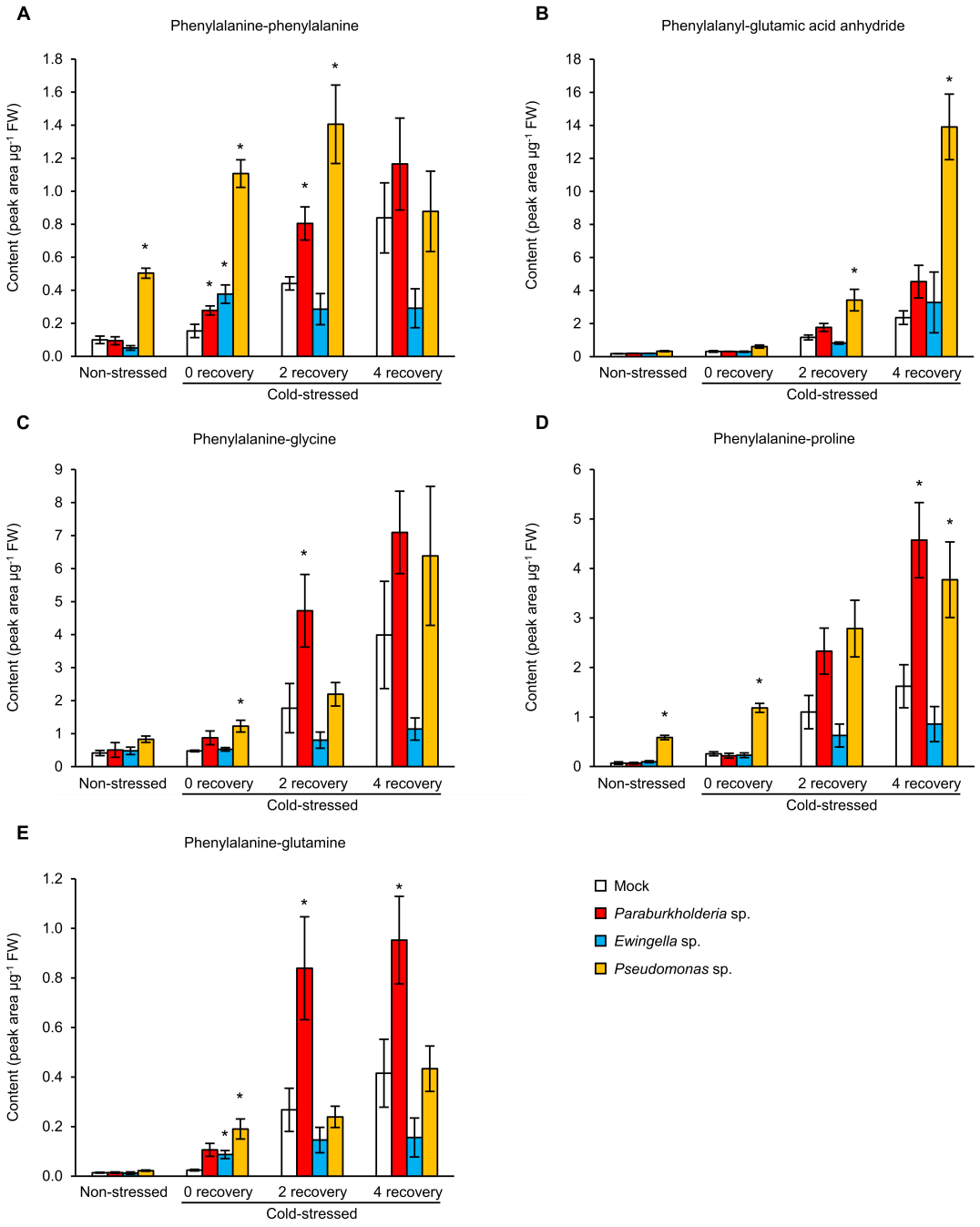

FIGURE 3 Content of phenylalanine-derived dipeptides in tomato samples. Tomato seeds were treated with sterile 10 mM $MgSO_4$ (mock-inoculated; white bars) or inoculated with *Paraburkholderia phytofirmans* PsJN (red bars), *Ewingella* sp. S1.OA.A_B6 (blue bars), and *Pseudomonas* sp. S2.OTC.A_B10 (yellow bars). Plants were exposed or not (non-stressed; 25 ± 1°C) to cold stress (cold-stressed; 4 ± 1°C) and collected immediately after cold stress exposure (0 recovery), or after two (2 recovery) and four (4 recovery) days of recovery at 25 ± 1°C. The content of compounds annotated as putative phenylalanine-phenylalanine (A), putative phenylalanyl-glutamic acid anhydride (B), putative phenylalanine-glycine (C), putative phenylalanine-proline (D), and putative phenylalanine-glutamine (E) was assessed by high-performance liquid chromatography-high resolution mass spectrometry analysis and expressed as peak area per µg of fresh weight (peak area μg^{-1} FW). Mean and standard error values of at least four replicates (a pool of five plants each) are reported for each inoculum condition and temperature regime. Asterisks indicate significant differences in metabolite content between bacterium-inoculated and mock-inoculated plants for each temperature regime, according to the Mann–Whitney test ($P \leq 0.05$).

cold stress mitigation. In particular, the content of salicylic acid, 4-hydroxybenzoic acid, five phenylalanine-derived dipeptides (putative phenylalanine-phenylalanine, putative phenylalanyl-glutamic acid anhydride, putative phenylalanine-glycine, putative phenylalanine-proline, and putative phenylalanine-glutamine), and three phenylalanine-derived compounds (putative vanilloloside, putative oxidized form vanilloyl hexose, and putative sinapate) was higher in bacterium-inoculated compared to mock-inoculated samples under cold stress, indicating that the accumulation of these compounds is stimulated by psychrotolerant endophytic bacteria.

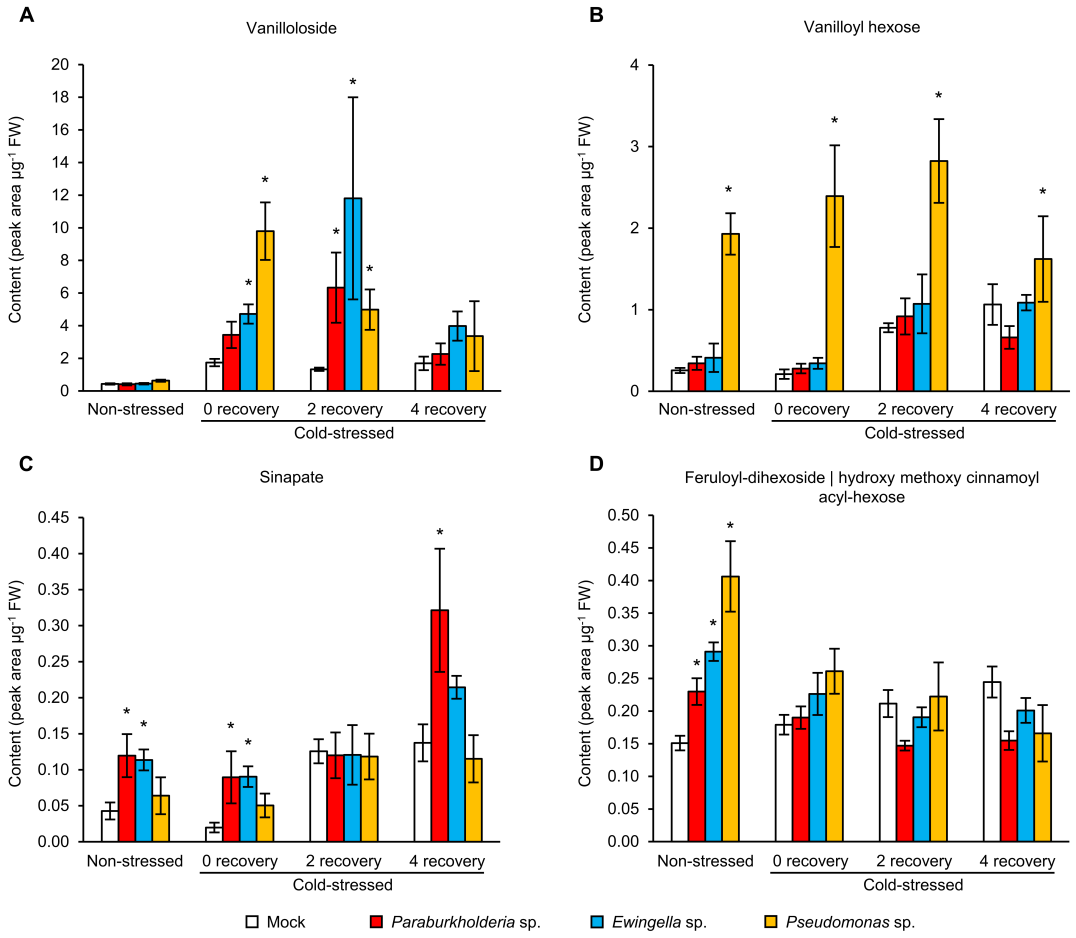

FIGURE 4 Content of phenylalanine-derived compounds in tomato samples. Tomato seeds were treated with sterile 10 mM $MgSO_4$ (mock-inoculated; white bars) or inoculated with *Paraburkholderia phytofirmans* PsJN (red bars), *Ewingella* sp. S1.OA.A_B6 (blue bars), and *Pseudomonas* sp. S2.OTC.A_B10 (yellow bars). Plants were exposed or not (non-stressed; 25 ± 1°C) to cold stress (cold-stressed; 4 ± 1°C) and collected immediately after cold stress exposure (0 recovery), or after two (2 recovery) and four (4 recovery) days of recovery at 25 ± 1°C. The content of compounds annotated as putative vanilloloside (A), putative vanilloyl hexose (B), putative sinapate (C), and putative feruloyl-dihexoside or hydroxy methoxy cinnamoyl acyl-hexose (D) was assessed by high-performance liquid chromatography-high resolution mass spectrometry analysis and expressed as peak area per µg of fresh weight (peak area μg^{-1} FW). Mean and standard error values of at least four replicates (a pool of five plants each) are reported for each inoculum condition and temperature regime. Asterisks indicate significant differences in metabolite content between bacterium-inoculated and mock-inoculated plants for each temperature regime, according to the Mann–Whitney test ($P \leq 0.05$).

For example, the bacteria-stimulated accumulation of salicylic acid (*Ewingella*, *Paraburkholderia*, and *Pseudomonas* inoculation), putative phenylalanine-phenylalanine (*Ewingella*, *Paraburkholderia*, and *Pseudomonas* inoculation), 4-hydroxybenzoic (*Ewingella* inoculation), putative vanilloloside (*Ewingella* and *Pseudomonas* inoculation), and putative sinapate (*Paraburkholderia* and *Ewingella* inoculation) was mainly found immediately after cold stress exposure (0 recovery), indicating a possible role in the cold stress mitigation. Although the involvement of phenylalanine-phenylalanine, vanilloloside, and sinapate was not previously reported under cold stress, exogenous application of 4-hydroxybenzoic acid is known to increase freezing tolerance in spring wheat (Horváth et al. 2007). Likewise, exogenous application of salicylic acid (or methyl salicylate) can enhance cold tolerance in different plant species (Miura and Tada 2014), such as tomato plants (Senaratna et al. 2000; Orabi et al. 2015; Meena et al. 2018) and tomato fruits (Ding et al. 2001, 2016; Zhang et al. 2011; Aghdam et al. 2014). In particular, salicylic acid can stimulate tomato cold tolerance through the modulation of cold stress-related genes, the activation of antioxidant enzymes (Ding et al. 2016), and the protection of photosystem II (Zhang et al. 2023). Furthermore, the bacteria-stimulated accumulation of putative phenylalanyl-glutamic acid anhydride (*Pseudomonas* sp. inoculation), putative phenylalanine-glycine (*Paraburkholderia* sp. inoculation), putative phenylalanine-glutamine (*Paraburkholderia* sp. inoculation), putative phenylalanine-proline (*Paraburkholderia* sp. and *Pseudomonas* sp. inoculation), and putative vanilloyl hexose (*Pseudomonas* sp. inoculation) was mainly observed after two and/or four days of recovery, indicating a potential role of these compounds in the re-adaptation processes after cold stress exposure. Although the role of these phenylalanine-derived dipeptides and other phenylalanine-derived compounds was not yet investigated under cold stress, they seem to be associated with plant stress responses under the conditions studied here. In particular, dipeptides are known to interact with target proteins and may serve regulatory functions, affecting cellular processes reflected in changes in plant growth and

stress tolerance (Minen et al. 2023). For example, it has been reported that the content of phenylalanine-derived dipeptides (e.g., glycine-phenylalanine, phenylalanine-valine/valine-phenylalanine) increased in root exudates of Arabidopsis plants after activation of stress-responsive mitogen-activated protein kinase cascades, suggesting a possible role of dipeptides in plant nutrition and plant–plant communication (Strehmel et al. 2017). Phenylalanine-derived dipeptides (e.g., phenylalanine-valine/valine-phenylalanine, phenylalanine-aspartate/aspartate-phenylalanine) are also known as products of proteolytic degradation in wheat plants treated with a *Fusarium* mycotoxin (deoxynivalenol) (Doppler et al. 2019b), indicating their possible involvement in response to biotic stresses. Moreover, phenylalanine-derived compounds (e.g., vanilloloside) are known to display antioxidant and antimicrobial activities (Sarikahya et al. 2011), suggesting a role in oxidative stress mitigation. Moreover, the content of chlorogenic acid and rutin was lower in bacterium-inoculated compared to mock-inoculated samples, indicating that psychrotolerant endophytic bacteria cause complex metabolic reprogramming in tomato plants exposed to cold stress. However, a large fraction of unknown compounds and features with multiple putative annotations was found in our study, indicating that further metabolomic studies with the use of chemical standards are required to reliably identify the biochemical constituents affected by bacterium inoculation in cold-stressed tomato plants. Further functional studies are also required to better clarify the role of phenylalanine-derived dipeptides and phenylalanine-derived compounds in cold stress mitigation.

5 | CONCLUSIONS

Cold stress is one of the most common abiotic factors limiting plant growth and crop production. Tomato seed inoculation with psychrotolerant endophytic bacteria reduced lipid peroxidation under cold stress and possible mitigation processes included a complex reprogramming of the metabolism of phenolic compounds. In particular, bacterial inoculation stimulated the metabolism of phenylalanine, ferulic acid, and p-coumaric acid in cold-stressed tomato plants, and increased the accumulation of secondary metabolites possibly involved in cold stress mitigation (e.g., 4-hydroxybenzoic and salicylic acid) and plant stress responses (e.g., phenylalanine-derived dipeptides). Although further metabolomic and functional studies are required to verify compound annotations and to better clarify the role of phenylalanine-derived compounds and phenylalanine-derived dipeptides in cold stress mitigation, these results provided a better understanding of metabolic changes stimulated by psychrotolerant endophytic bacteria in cold-stressed tomato plants. Thus, the validation of cold stress mitigation activated by psychrotolerant endophytic bacteria on potted tomato plants will pave the way for the further development of endophytic bacterial inoculants as sustainable products to protect crops against cold stress.

AUTHOR CONTRIBUTIONS

GL and CS carried out the experiments on tomato plants. GL, MD, CB, and DR carried out the metabolomics measurements, subsequent data evaluation, and metabolite annotations. GL, RS, and MP contributed to data interpretation. MP and RS conceived the study, designed the experiments, and coordinated all research activities. GL, RS, and MP wrote the manuscript. All the authors revised and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supporting information of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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