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Screening of As-Resistant Bacterial Strains from the Bulk Soil and the Rhizosphere of Mycorrhizal *Pteris vittata* Cultivated in an Industrial Multi-Polluted Site

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Abstract: Arsenic (As) contamination poses significant environmental and health concerns globally, particularly in regions with high exposure levels due to anthropogenic activities. As phytoremediation, particularly through the hyperaccumulator fern *Pteris vittata*, offers a promising approach to mitigate arsenic pollution. Bacteria and mycorrhizal fungi colonizing *P. vittata* roots are involved in As metabolism and resistance and plant growth promotion under stressful conditions. A total of 45 bacterial strains were isolated from bulk soil and the rhizosphere of mycorrhizal *P. vittata* growing in an industrial As-polluted site. Bacteria were characterized by their plant-beneficial traits, tolerance to sodium arsenate and arsenite, and the occurrence of As-resistant genes. This study highlights differences between the culturable fraction of the microbiota associated with the rhizosphere of mycorrhizal *P. vittata* plants and the bulk soil. Moreover, several strains showing arsenate tolerance up to 600 mM were isolated. All the bacterial strains possessed *arsC* genes, and about 70% of them showed *arrA* genes involved in the anaerobic arsenate respiration pathway. The possible exploitation of such bacterial strains in strategies devoted to the assisted phytoremediation of arsenic highlights the importance of such a study in order to develop effective in situ phytoremediation strategies.

Keywords: arsenic; hyperaccumulators; plant growth promotion; mycorrhizosphere; arsenic resistance

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1. Introduction

Arsenic (As) is widely distributed throughout environmental components, such as air, water and soil, with a mean concentration of 3 mg kg^{-1} As and is ranked 20th in the Earth's crust [1]. In soil, the natural As content ranges from 0.2 to about 40 mg kg^{-1} [2]; in polluted sites, such as copper-smelting plants and pesticide-contaminated agricultural soils, its concentration can reach 100–2500 mg kg^{-1} (the World Health Organization [WHO], 2000).

Arsenic can also be derived from natural processes and can be released by rock weathering, volcanic emissions and discharges from hot springs [3]. However, the amount of As introduced into the environment increases through human activities, such as mining, smelting, forest wildfires and the use of pesticides and herbicides [4–6]. As a result, in 2017, the US-EPA counted more than 600 As-contaminated sites in the US requiring remediation processes (US-EPA, 2017). Arsenic pollution represents a major issue, especially in Europe, Bangladesh, Taiwan, India, Malaysia, Vietnam, China, Mexico and Pakistan; the worst situation is in South and Southeast Asia, where millions of people are exposed to high levels of arsenic in drinking water [7,8]. Here, arsenic represents a potential health risk to the environment and human health due to its toxicity and carcinogenicity, especially

in its inorganic forms. In the environment, arsenate (AsV) and arsenite (AsIII) are the most abundant inorganic forms of As. Both of them show high toxicity in microorganisms, plants and mammals but differ in their availability [9]. In fact, arsenate is characterized by an elevated mobility in the water ecosystem and an important affinity for the sulfhydryl groups that are part of cellular enzymes, thus leading to higher toxicity compared to arsenite. While under anaerobiosis, microorganisms may use arsenate as an electron acceptor during anaerobic respiration, and in an aerobic environment, microorganisms can catalyze the reduction of arsenate to arsenite that is extruded out of the cell through a specific arsenite transporter [10,11]. Therefore, microorganisms that are able to transform arsenate to arsenite play a crucial role in the modulation of As toxicity in the environment.

Although arsenic is toxic for the majority of plant species, hyperaccumulator plants are able to store heavy metals and metalloids up to 1% of their dry weight without showing toxicity-related symptoms [12]. Usually, plants classified as hyperaccumulators uptake arsenate or arsenite and transport them to their aboveground parts, where As is then stored, allowing contaminated soils to be cleaned up and avoiding arsenic accumulation through the food chain [13]. In 2001, the first As hyperaccumulator, the Chinese brake fern (*Pteris vittata*), with the capability to accumulate up to 2–3% of As in its fronds and tolerate arsenic concentrations of up to 1500 ppm, was discovered [14]. Since then, the mechanisms by which *P. vittata* can tolerate, detoxify and accumulate arsenic have been widely analyzed and well characterized [15–19]. Amongst plant-beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) can establish symbiosis with most terrestrial plants, including ferns, such as *P. vittata*, leading to increased plant biomass and plant nutrition improvement, modulating arsenate transfer to the fronds and enhancing plant tolerance to stresses [20–24]. The increased arsenic accumulation in the fronds observed in mycorrhizal *P. vittata* is possibly related to the upregulation of a putative arsenic transporter, PgPOR29, following plant inoculation with AMF [18].

In this context, exploring the cultivable fraction of this rhizosphere microbiota is crucial, as these bacteria hold promise for application in phytoremediation technologies, further underlining the importance of studying the entire rhizosphere ecosystem for advancing sustainable bioremediation strategies. The role of bacteria colonizing the roots of this fern in supporting plant growth in such a hostile environment and As metabolism has been less investigated. A quick search of the literature database (WoS) performed in April 2024 by cross-referencing the keywords *Pteris vittata* AND bacteria resulted in 119 papers since 2006, thus demonstrating that there is still a need for new information in this context. Moreover, to our knowledge, no published paper focused on the rhizosphere of mycorrhizal *P. vittata* growing in a multi-metal contaminated industrial site. The first paper on this topic was published in 2010 by the group of Lena Ma, who isolated and identified 20 bacterial strains from the rhizosphere of *P. vittata* tolerating 400 mmol/L of arsenate and metabolizing it to arsenite in a broth medium [23]. The occurrence of bacterial endophytes has also been explored [25–30]. Most of the papers reporting the efficacy of rhizospheric or endophytic bacterial strains in plant growth promotion or metabolizing arsenic were performed under controlled conditions in pot experiments on As-amended soils [27,28,31,32]. In contrast, only a very low number of articles deal with experiments performed directly in polluted sites [29,33,34].

During 2011–2014, we participated in a project focused on the soil remediation of an industrial site (at that time, Solvay Solexis S.p.a.) located in the north-west of Italy, characterized by a multiplicity of mainly inorganic pollutants, including arsenic. Two experimental fields (10 × 10 m and 60 × 1.5 m) were set up with 231 and 357 plants of mycorrhizal *P. vittata*, respectively. The As-remediation efficiency of these mycorrhizal *P. vittata* was evaluated after three years of growth in this area and well described by Cantamessa et al. [24]. In order to select microorganisms able to improve arsenic phytoremediation by *P. vittata*, we isolated, identified and characterized their plant-beneficial physiological features (phosphate solubilization, siderophore release and auxin production), the traits involved in arsenic metabolism and the bacterial strains living in the bulk soil and

the rhizosphere of mycorrhizal *P. vittata* cultivated for three years in this multi-polluted industrial site.

2. Materials and Methods

2.1. Soil Sampling

The map of the industrial site (44°53′15″ N 8°40′07″ E) and the sampling point are reported in Figure 1.

Figure 1. GPS image of the industrial site: the blue label indicates the experimental field where mycorrhizal *P. vittata* was cultivated (44°53′15″ N 8°40′07″ E). The site is located in northwestern Italy and is polluted by heavy metals due to the metallurgic planting facility's activities.

Bulk soil and the soil associated with the roots of mycorrhizal *P. vittata* were collected at a depth of 30 cm (topsoil) after removing the surface layer (3.0–5.0 cm). Three soil cores (about 1 kg) were taken in the proximity of five ferns and from five points in the unplanted soil. The roots entrapped in the soil cores collected 3 cm close to the ferns were assessed for mycorrhizal colonization. Briefly, the roots were fixed in 70% ethanol and cut into 1 cm-long pieces. The roots were then cleared with 10% KOH for 45 min at 60 °C, stained with 1% methyl blue in lactic acid and mounted on a slide. The evaluation of mycorrhizal colonization was measured, as described in 1986 by Trouvelot et al. [35], and plants reaching up to 50% were considered for the sampling of the mycorrhizosphere soil. The soil adhering to these roots was removed using sterile gloves. As recommended by the Italian law GU 179/2002, for soil microbiological characterization analysis, the three subsamples of mycorrhizosphere or bulk soil were pooled to obtain homogeneous samples. The soil samples were then immediately processed in the laboratory for bacterial isolation. The workflow is represented in Figure 2.

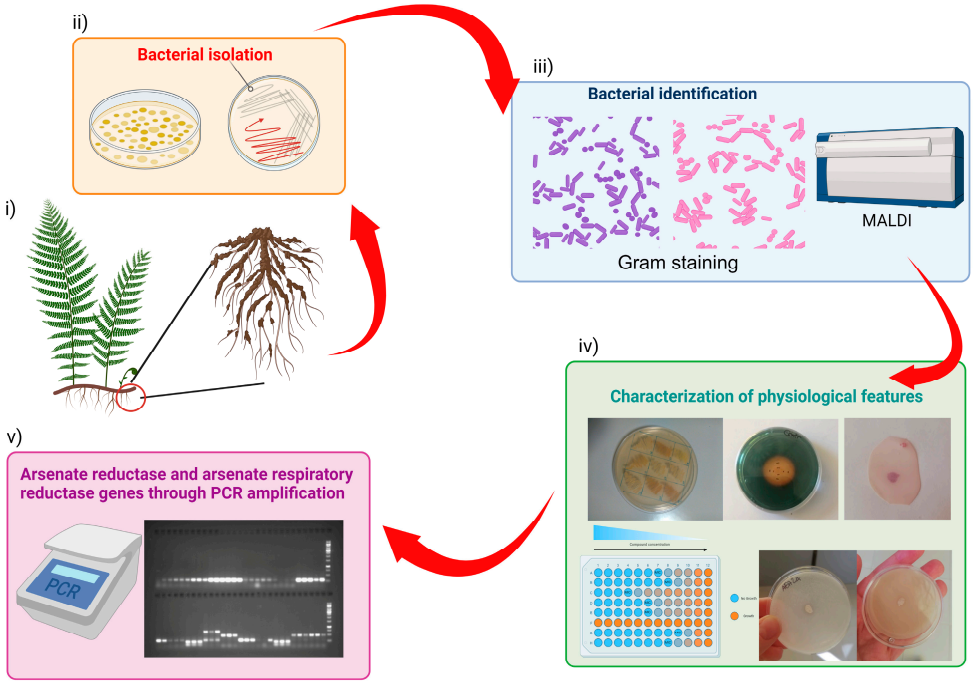


Figure 2. Image describing the different stages of the experimental procedure used: (**i**) soil sampling from the roots of *P. vittata* cultivated in the industrial site; (**ii**) bacterial isolation on agar plates; (**iii**) Gram staining and identification of the selected strains via MALDI (Matrix-152 Assisted Laser Desorption/Ionization) and TOF/TOF (UltrafleXtreme, Bruker) system; (**iv**) characterization of plant beneficial physiological traits and determination of the arsenite and arsenate tolerance of the bacterial strains; (**v**) occurrence of arsenate reductase and arsenate respiratory reductase genes assessed by PCR amplification. This image was created with BioRender (https://www.biorender.com, accessed on 30 July 2024), Toronto, Canada.

2.2. Isolation and Extraction of Culturable Bacteria

The isolation of culturable bacterial strains from the mycorrhizosphere and bulk soil was performed, as reported by Novello et al. (2023) [36]. Briefly, ten grams of fresh soil were added to 90 mL of $MgSO_4$-$7H_2O$ (0.1 M, Sigma-Aldrich, Milano, Italy), shaken at room temperature at 180 rpm for 1 h and allowed to sediment for 30 min. Serial dilutions (until 10^{-4}) were performed in magnesium sulfate buffer, and 100 µL of each suspension was distributed on 10% Tryptic Soy Agar (TSA, Sigma-Aldrich) with 100 µg mL^{-1} of cycloheximide added as an antifungal agent (Sigma-Aldrich). Sodium arsenate (10 mM, Sigma-Aldrich) was also added to half of the plates. The bacterial density was recorded after 2 and 7 days of incubation at 28 °C and expressed as log CFU g^{-1} of dry soil. Twenty to thirty colonies from a representative dilution were selected and isolated on TSA plates. Cultures of bacterial cells, grown in TSB (Biolife, Monza, Italy), were stored in 50% glycerol at −80 °C. All isolated strains were subjected to Gram staining and a description of the colony morphology with a stereomicroscope.

Data regarding bacterial densities were statistically compared by one-way ANOVA using StatView Software, ver. 4.5 (Abacus Concepts; Berkeley, CA, USA). Differences were considered significant for p-values < 0.05.

2.3. Identification of Bacterial Strains

The identification of bacterial strains was conducted using MALDI (Matrix-Assisted Laser Desorption/Ionization) and TOF-TOF (UltrafleXtreme, Bruker, Billerica, MA, USA) mass spectrometry, following the procedure outlined by Novello et al. (2023) [36]. A freshly cultured bacterial colony on TSA was applied in triplicate onto an MTP 384 target plate (Bruker Daltonics, Milan, Italy). Initially, the spot was treated with 70% formic acid (Sigma-Aldrich, Burlington, MA, USA) and subsequently with alpha-cyano-4-hydroxycinnamic

acid (HCCA) (Bruker, Milan, Italy). The target plate was allowed to air-dry at room temperature until the sample crystallized. The acquired mass spectra for each bacterial strain were subjected to analysis by Biotyper software v.20 (Bruker Daltonics, Milan, Italy) and referred to standard matching.

2.4. Exploring Plant-Beneficial Physiological Traits and Temperature Test

The bacterial strains were characterized for the physiological activities underlying their plant growth-promoting effect (such as phosphate solubilization, IAA synthesis and siderophore production) and were tested for their ability to grow at different temperatures (4, 28, 37 and 42 °C) [36].

Their ability to solubilize phosphate was evaluated by spotting the isolates in the center of plates containing two growth media, one containing tricalcium phosphate (TCP) and another one containing dicalcium phosphate (DCP). Plates were incubated for 15 days at 28 °C, and phosphate solubilization was highlighted with colony growth for the TCP plates and colony growth with a clarification halo for the DCP plates.

The synthesis and production of IAA (indole-3 acetic acid) were evaluated on 10% TSA plates containing 5 mM of tryptophan (Sigma-Aldrich, Burlington, MA, USA), as reported by De Brito Alvarez et al. (1995) [37]. Single colonies were inoculated in the center of three plates, covered with nitrocellulose disks and incubated at 28 °C for 72 h. At the end of the incubation, the disks were removed and reacted with Salkowski's reagent (0.5 M of $FeCl_3$ in 35% $HClO_4$) for 1–3 h. The presence of a red/pink halo indicated the synthesis of IAA.

For the siderophore release assessment, three Petri dishes containing the universal Chrome Azurol S (CAS) medium were inoculated in the center with each bacterial strain and incubated at 28 °C for 7 days [38]. The production of siderophores was evidenced by the presence of an orange halo around the colony and reported as the ratio between the diameter of the halo and that of the colony.

A temperature test was performed in order to evaluate the growth range of the bacterial isolates. TSA plates were spotted with a colony of each bacterial strain and incubated at four different temperatures for 24–48 h.

2.5. Assessment of Arsenate and Arsenite Effect by Minimal Inhibitory Concentration

The bacterial tolerance to arsenate and arsenite was assessed by determining the MIC (Minimum Inhibitory Concentration) using the microdilution method. Sodium arsenate (Sigma-Aldrich, Milan, Italy) and sodium arsenite (Sigma-Aldrich) were dissolved in TSB to obtain a final starting concentration of 600 mM and 4 mM in the plates, respectively. The assay was performed in 96-well plates. The starting solution was then serially diluted (2:1) to obtain a range from 600 mM to 0.58 mM for arsenate and from 4 mM to 0.007 mM for arsenite. A fresh culture of each bacterium was diluted in $MgSO_4$ buffer (0.1 M) up to a concentration of 10^8 CFU/mL, then diluted in TSB to a final concentration of 10^5 CFU/mL. This final suspension was added to each well. A positive control, represented by TSB with a bacteria inoculum, and a negative control, containing TSB with arsenate and arsenite, were carried out. The 96-well plates were incubated at 28 °C for 24/48 h and considered positive when turbidity was visible. Each experiment was performed in triplicate.

2.6. Arsenate Reductase and Arsenate Respiratory Reductase Gene PCR Amplifications

The arsC (arsenate reductase) and arrA (arsenate respiratory reductase) genes were targeted using six and three primer sets, respectively, as shown in Table 1 and previously described by Escudero et al. (2013) [39]. Two of the six primer sets amplify glutaredoxin-dependent arsC, while the other four amplify thyroxine-dependent arsC.

All PCR reactions were prepared as follows: Genomic DNA at different dilutions (1:1, 1:10 and 1:50) was used for gene amplifications. The reactions were performed in a final volume of 20 µL containing 2 µL of 10× PCR buffer (containing 15 mM of $MgCl_2$) (Finnzymes, Woburn, MA Finland); 500 µM of dNTPs (125 µM of each dNTP); 500 nM of each primer; and 0.02 U μL^{-1} of Taq DNA polymerase (Finnzymes). In the amlt-42-

F/amlt-376-R/smrc-42-F/smrc-376-R primer mix, 250 nM of each primer was used. Five ml of diluted or undiluted genomic DNA wereas added to 15 mL of the PCR mix, and the amplifications were performed in a thermal cycler (Techne, Bibby Scientific, Segrate, Milan, Italy). The PCR primer set programs are listed in Table 2. In order to enhance the efficiency of the amplification and increase the amount of DNA for the arsenate respiratory reductase arrA gene, a hemi-nested PCR was carried out using the primer pairs AS1R and AS1R for the first amplification step and AS2F and AS1R for the second one. The PCR products were separated by gel electrophoresis on a 1.2% agarose gel in TAE buffer, and DNA was visualized under UV light after being stained with ethidium bromide.

Table 1. Primer sets used for arsenate reductase and arsenate respiratory reductase gene PCR amplifications.

	Primer Set	Primer Name	Primer Sequence (5′–3′)
Arsenate reductase	arsC-Grx-Sun	amlt-42-F	TCG CGT AAT ACG CTG GAG AT
		amlt-376-R	ACT TTC TCG CCG TCT TCC TT
		smrc-42-F	TCA CGC AAT ACC CTT GAA ATG ATC
		smrc-376-R	ACC TTT TCA CCG TCC TCT TTC GT
	arsC-Grx-Saltikov	Q-arsC-F1	GAT TTA CCA TAA TCC GGC CTG T
		Q-arsC-R1	GGC GTC TCA AGG TAG AGG ATA A
	arsC-Trx-Villegas	arsCGP-Fw	TGC TG ATTT AGT TGT TAC GC
		arsCGP-Rv	TTC CTT CAA CCT ATT CCC TA
	arsC-Trx1a	arsC 4F	TCH TGY CGH AGY CAA ATG GCH GAA G
		arsC 4R	GCN GGA TCV TCR AAW CCC CAR TG
	arsC-Trx1b	arsC 5F	GGH AAY TCH TGY CGN AGY CAA ATG GC
		arsC 5R	GCN GGA TCV TCR AAW CCC CAR NWC
	arsC-Trx2	arsC 6F	CAC VTG CMG RAA DGC RAR RVV DTG GCTCG
		arsC 6R2	TTR WAS CCN ACG WTA ACA KKH YYK YC
Arsenate respiratory reductase	arrA1	arrA F	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYT T
		arrA R	CCT GTG ATT TCA GGT GCC CAY TY V GGN GT
	arrA2	AS1 F	CGA AGT TCG TCC CGA THA CNT GG
		AS1 R	GGG GTG CGG TCY TTN ARY TC
		AS2 F	GTC CCN ATB ASN TGG GAN RAR GCN MT
	arrA3	HAArrA-D1F	CCG CTA CTA CAC CGA GGG CWW YTG GGR NTA
		HAArrA-G2R	CGT GCG GTC CTT GAG CTC NWD RTT CCA CC

Table 2. PCR amplification program details for specific set of primers.

ArsC 4F/ArsC 4R	ArsC 5F/ArsC 5R	ArsC 6F/ArsC 6R2	ArsCGP-Fw/ ArsCGP-Rv	Q-arsC-F1/Q-arsC-R
95 °C 5′	95 °C 5′	95 °C 5′	95 °C 5′	95 °C 5′
95 °C 1′ 46.7 °C 1′ 40 cycles 72 °C 50″	95 °C 1′ 60 °C 1′ 40 cycles 72 °C 50″	95 °C 1′ 54.5 °C 1′ 40 cycles 72 °C 50″	95 °C 1′ 48 °C 1′ 40 cycles 72 °C 50″	95 °C 1′ 60 °C 1′ 40 cycles 72 °C 50″
72 °C 10′	72 °C 10′	72 °C 10′	72 °C 10′	72 °C 10′
NESTED-PCR n1 AS1 F/AS1 R	NESTED-PCR n2 AS2 F/AS1 R	HAArrA-D1 F/HAArrA-G2 R	arrA F/arrA R	amlt-42-F/amlt-376-R/smrc-42-F/smrc-376-R mix
95 °C 5′	95 °C 5′	95 °C 5′	95 °C 5′	95 °C 5′
95 °C 1′ 50 °C 1′ 35 cycles 72 °C 1′	95 °C 1′ 55 °C 1′ 30 cycles 72 °C 1′	95 °C 1′ 53.5 °C 1′ 40 cycles 72 °C 1′	95 °C 1′ 50 °C 1′ 40 cycles 72 °C 30″	95 °C 1′ 60 °C 1′ 40 cycles 72 °C 50″
72 °C 10′	72 °C 10′	72 °C 10′	72 °C 10′	72 °C 10′

3. Results

3.1. Quantification of Culturable Bacteria

The bacterial density of the culturable fraction isolated from the contaminated area was evaluated as log CFU g^{-1} of dry soil. The cultivable bacterial density in the mycorrhizosphere was higher than that recorded in the bulk soil (PTV vs. BS $p = 0.02$). In detail, the bacterial density was 4.59 in PTV (total bacteria from the mycorrhizosphere of *P. vittata*), 4.50 in PTV-As (As-tolerant bacteria from the mycorrhizosphere of *P. vittata*) and 4.14 in BS (total bacteria from the bulk soil) and BS-As (As-tolerant bacteria from the bulk soil). The fraction of Gram-positive bacteria was 60% in BS, 30% in Bs-As, 73% in PTV and 67% in PTV-As. Consequently, the highest amount of Gram-negative isolates was found in the BS-As sample (70%). In all the samples, a prevalence of *r*-strategist, fast-growing strains was observed: 79.49% in PTV, 81.25% in PTV-As and 78.57% in BS and BS-As.

3.2. Identification of Bacterial Strains

A total of 45 strains were selected, identified, characterized by their physiological traits and subjected to molecular analysis in order to identify possible genes involved in As tolerance. Twenty colonies were isolated from the *P. vittata* mycorrhizosphere: eleven were selected from a culture medium without arsenic and nine from the same medium with added sodium arsenate. Another 25 colonies were selected from the bulk soil: 15 strains were isolated on a culture medium without arsenic and 10 from the same medium with added sodium arsenate (Table 3).

Table 3. Identification and physiological characterization (growth temperature, siderophore production, phosphate solubilization and synthesis of auxin) of strain isolated from mycorrhizosphere of *P. vittata* (PTV, without added As and PTV-As, with added As) and bulk soil (BS, without added As and BS-As, with added As).

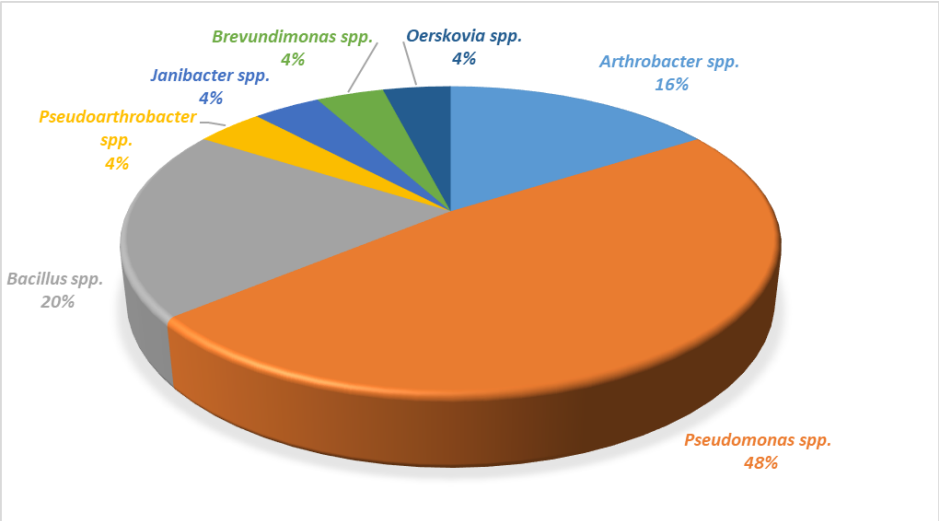
Strain	Taxonomy Identification	Growth 4 °C	Growth 28 °C	Growth 37 °C	Growth 42 °C	CAS *	DCP/TCP #	IAA @
BS1	*Arthrobacter* sp.	+	+	+	-	2.8	0	0
BS3	*Arthrobacter* sp.	+	+	+	+	0	0	0
BS5	*Bacillus* sp.	+	+	+	-	0	0	0
BS6	*Bacillus* sp.	+	+	+	±	0	0	1
BS7	*Pseudomonas* sp.	+	+	-	-	2.4	0	0
BS8	*Pseudoarthrobacter oxydans*	+	+	+	-	0	0	0
BS9	*Pseudomonas* sp.	+	+	-	-	2.8	0	0
BS10	*Pseudomonas marginalis*	+	+	+	+	2.5	0	2
BS11	*Janibacter* sp.	+	+	+	-	0	0	1
BS12	*Arthrobacter* sp.	+	+	+	-	0	0	0
BS15	*Bacillus* sp.	+	+	-	-	0	0	0
BS16	*Bacillus* sp.	+	+	+	--	0	0	0
BS18	*Pseudomonas brassicacearum*	+	+	-	-	1.9	0	0
BS19	*Pseudomonas thivervalensis*	+	+	-	-	3.4	0	2
BS20	*Pseudomonas* sp.	+	+	+	-	3.1	0	0
BS-As2	*Arthrobacter* sp.	+	+	+	-	0	0	2
BS-As3	*Pseudomonas* sp.	+	+	+	+	2.3	0	0
BS-As6	*Pseudomonas* sp.	-	+	+	-	0	0	0
BS-As7	*Pseudomonas marginalis*	+	+	-	-	0	0	0
BS-As11	*Bacillus* sp.	+	+	+	+	0	0	1
BS-As12	*Brevundimonas diminuta*	+	+	+	±	0	0	0
BS-As13	*Pseudomonas* sp.	-	+	+	-	1.8	0	0
BS-As14	*Oerskovia* sp.	+	+	-	-	1.4	0	0
BS-As19	*Pseudomonas marginalis*	+	+	+	-	2.7	0	2
BS-As22	*Pseudomonas marginalis*	+	+	+	--	0	0	0
PTV7	*Janibacter* sp.	-	+	+	+	0	0	0
PTV9	*Microbacterium* sp.	+	+	+	+	0	0	0
PTV15	*Bacillus megaterium*	± §	+	+	+	1.1	0	3

Table 3. *Cont.*

Strain	Taxonomy Identification	Growth 4 °C	Growth 28 °C	Growth 37 °C	Growth 42 °C	CAS *	DCP/TCP #	IAA @
PTV18	*Pedobacter* sp.	+	+	+	±	0	0	0
PTV20	*Brevundimonas diminuta*	+	+	+	±	0	0	0
PTV21	*Bacillus* sp.	+	+	+	+	1.4	0	0
PTV22	*Bacillus cereus*	--	+	+	+	0	0	0
PTV23	*Bacillus pumilus*	-	+	+	+	1.2	0	0
PTV25	*Janibacter* sp.	+	+	+	+	0	0	0
PTV28	*Brevundimonas diminuta*	-	+	+	+	0	0	0
PTV30	*Bacillus thuringensis*	-	+	+	+	0	0	0
PTV-As3	*Micrococcus* sp.	-	+	+	--	0	0	0
PTV-As5	*Brevundimonas diminuta*	-	+	+	-	0	0	1
PTV-As7	*Brevundimonas diminuta*	-	+	+	-	0	0	2
PTV-As8	*Bacillus* sp.	-	+	+	-	3.3	0	2
PTV-As9	*Janibacter* sp.	+	+	+	+	2.3	0	0
PTV-As15	*Bacillus* sp.	-	+	+	+	0	0	0
PTV-As23	*Bacillus pumilus*	+	+	+	±	2.9	0	1
PTV-As26	*Bacillus* sp.	+	+	+	-	0	0	0
PTV-As29	*Pseudomonas marginalis*	+	+	+	±	2.5	0	0

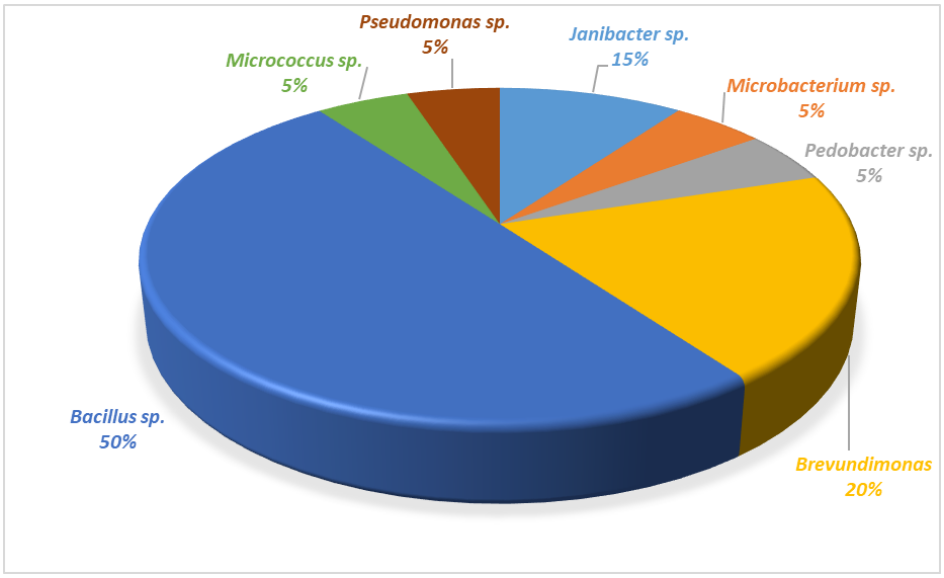
§ The use of the symbol ± stands for limited growth. * CAS: Siderophore production was expressed as the ratio between the two diameters of the halo and the two diameters of the colony. # Phosphate solubilization. DCP = Dicalcium phosphate and TCP = Tricalcium phosphate. @ Synthesis of IAA was expressed as intensity color scale (0 = no color, 1 = light pink, 2 = pink, 3 = light red, 4 = red).

The most representative genus was *Bacillus*, followed by *Pseudomonas*, *Brevundimonas* and *Janibacter* (31%, 27%, 11% and 9% of the total, respectively). The *Bacillus* genus was more frequent in the *P. vittata* mycorrhizosphere (45%) than in the bulk soil (20%). An opposite situation was observed for the *Pseudomonas* genus, which was dominant in the bulk soil (48%) and less spread in the mycorrhizosphere (5%). Bacteria belonging to the *Arthrobacter* genus (four strains, with 16% of the strains isolated from BS and BS-As) occurred only in the bulk soil (Table 3 and Figure 3A,B). Finally, the identification reveals the presence of only one isolate belonging to the *Microbacterium*, *Pedobacter*, *Micrococcus*, *Pseudoarthrobacter* and *Oerskovia* genera, representing 2% of the total identified isolates (Table 3).



(**A**)

Figure 3. *Cont.*



(**B**)

Figure 3. Distribution (frequency) of the bacterial genera found in bulk soil (**A**) and rhizosphere of mycorrhizal *P. vittata* (**B**) in the industrial site considered.

3.3. Characterization of Physiological Traits and Temperature Test

All the strains (45 bacterial strains) were tested for their ability to solubilize phosphate, produce auxins and synthesize siderophores. No phosphate-solubilizing bacteria were found either in the mycorrhizosphere soil or the bulk soil.

The results (Table 3) show that 40% (18 out of 45 strains) of the selected bacteria were able to produce siderophores. In detail, four of them were found in PTV-As and BS-As, three in the mycorrhizosphere of *P. vittata* (PTV) and seven in the bulk soil (BS). Auxin synthesis was detected in 26% of the strains (12 out of 45). Only 1 out of 12 were isolated from PTV, 4 from PTV-As, 4 from BS and 3 from BS-As. Bacteria able to synthesize siderophores were more abundant than IAA-producing bacteria: the capability to release iron-chelating molecules was more frequent in the bulk soil than in the mycorrhizosphere of *P. vittata* (44% vs. 35%). Only 6 strains out of 45, namely, *Bacillus megaterium* PTV15, *Bacillus* sp. PTV-As8, *Bacillus pumilus* PTV-As23, *Pseudomonas marginalis* BS10, *Pseudomonas thivervalensis* BS19 and *P. marginalis* BS-As19 were able to synthesize both IAA and siderophores. Finally, the temperature range for growth was assessed for each bacterial strain. All tested bacteria were able to grow at 28 °C, 71% (32 out of 45 strains) at 4 °C, 84% (38 out of 45) at 37 °C and only 33% (15 out of 45) at 42 °C. While all PTV and PTV-As isolated strains were able to grow at 37 °C, this capability was recorded in only 18 out of 25 (72%) of the strains isolated from the bulk soil.

3.4. Minimal Inhibitory Concentration of Arsenate and Arsenite

All the bacterial strains tolerated concentrations higher than 4 mM of sodium arsenite. Only one strain (BS1, identified as *Arthrobacter* sp.) was tolerant to more than 600 mM of sodium arsenate. For all other strains, the Minimal Inhibitory Concentration (MIC) values of sodium arsenate were as follows: 15 strains (33%) were tolerant up to 600 mM, 15 strains (33%) up to 300 mM, 10 strains (22%) up to 150 mM and 3 strains (6%) up to 75 mM. The MIC values of sodium arsenite and sodium arsenate for all the selected strains are reported in Table 4.

Table 4. Minimal Inhibitory Concentration (MIC) values of sodium arsenate and sodium arsenite for the 45 selected bacterial strains.

	Strain	Arsenate mM	Arsenite mM
Arthrobacter sp.	BS1	>600	>4
Arthrobacter sp.	BS3	600	>4
Bacillus sp.	BS5	300	>4
Bacillus sp.	BS6	300	>4
Pseudomonas sp.	BS7	300	>4
Pseudoarthrobacter oxydans	BS8	300	>4
Pseudomonas sp.	BS9	300	>4
Pseudomonas marginalis	BS10	150	>4
Janibacter sp.	BS11	300	>4
Arthrobacter sp.	BS12	300	>4
Bacillus sp.	BS15	150	>4
Bacillus sp.	BS16	300	>4
Pseudomonas brassicacearum	BS18	150	>4
Pseudomonas thivervalensis	BS19	150	>4
Pseudomonas sp.	BS20	150	>4
Arthrobacter sp.	BS-As2	600	>4
Pseudomonas sp.	BS-As3	600	>4
Pseudomonas sp.	BS-As6	150	>4
Pseudomonas marginalis	BS-As7	300	>4
Bacillus sp.	BS-As11	300	>4
Brevundimonas diminuta	BS-As12	600	>4
Pseudomonas sp.	BS-As13	150	>4
Oerskovia sp.	BS-As14	600	>4
Pseudomonas marginalis	BS-As19	75	>4
Pseudomonas marginalis	BS-As22	150	>4
Janibacter sp.	PTV7	600	>4
Microbacterium sp.	PTV9	ND *	>4
Bacillus megaterium	PTV15	75	>4
Pedobacter sp.	PTV18	75	>4
Brevundimonas diminuta	PTV20	600	>4
Bacillus sp.	PTV21	150	>4
Bacillus cereus	PTV22	150	>4
Bacillus pumilus	PTV23	600	>4
Janibacter sp.	PTV25	300	>4
Brevundimonas diminuta	PTV28	600	>4
Bacillus thuringensis	PTV30	300	>4
Micrococcus sp.	PTV-As3	600	>4
Brevundimonas diminuta	PTV-As5	600	>4
Brevundimonas diminuta	PTV-As7	600	>4
Bacillus sp.	PTV-As8	600	>4
Janibacter sp.	PTV-As9	600	>4
Bacillus sp.	PTV-As15	300	>4
Bacillus pumilus	PTV-As23	600	>4
Bacillus sp.	PTV-As26	300	>4
Pseudomonas marginalis	PTV-As29	300	>4

* ND = Not determined.

3.5. Arsenate Reductase and Arsenate Respiratory Pathways

The presence of arsC and arrA genes in the selected bacterial strains was determined by PCR and is reported in Table 5. Glutareroxin-dependent arsC genes were absent in all the bacterial isolates, while thyrodoxin-dependent arsC genes were detected in all the strains. In particular, all bacteria except PTV-As29, BS7, BS18, BS19, BS-As7, BS-As14 BS-As19 and BS-As22 were positive to primer set ArsC/ArsC 4R. The strain PTV-As29 was positive to the primer sets ArsC 6F/ArsC 6R2 and ArsCGP-Fw/ArsCGP-Rv. The isolates BS7, BS18, BS19, BS-As7 and BSAs14 were positive to the primer set ArsC 6F/ArsC 6R2, and BS-As19

and BS-As22 were positive to the primer sets ArsC 6F/ArsC 6R2 and ArsC 5F/ArsC 5R. All bacteria except PTV7, PTV9, PTV15, PTV18, PTV25, PTV28, PTV30, BS1, BS3, BS8, BS11, BS12, BS14, BS15, BS-As11 and BS-As12 had arsenate respiratory reductase genes.

Table 5. Occurrence of arsenate reductase and arsenate respiratory reductase gene in the selected bacterial strains.

	Strain	ArsC-Trx1a	ArsC-Trx1b	ArsC-Trx2	ArsC-Trx Villegas	arrA1 Ars-Respiratory	arrA2 Ars-Respiratory	arrA3 Ars-Respiratory
		Arsenate Reductase				Arsenate Respiratory Reductase		
Arthrobacter sp.	BS1	+	-	-	-	-	-	-
Arthrobacter sp.	BS3	+	-	-	-	-	-	-
Bacillus sp.	BS5	+	-	-	-	+	-	-
Bacillus sp.	BS6	+	-	-	-	+	+	-
Pseudomonas sp.	BS7	-	-	+	-	+	-	-
Pseudoarthrobacter oxydans	BS8	+	-	-	-	-	-	-
Pseudomonas sp.	BS9	+	-	+	-	+	-	-
Pseudomonas marginalis	BS10	-	-	+	+	+	+	-
Janibacter sp.	BS11	+	-	-	-	-	-	-
Arthrobacter sp.	BS12	+	-	-	-	-	-	-
Bacillus sp.	BS15	+	-	-	-	-	-	-
Bacillus sp.	BS16	+	-	-	-	-	-	-
Pseudomonas brassicacearum	BS18	-	-	+	-	+	+	-
Pseudomonas thivervalensis	BS19	-	-	+	-	+	-	-
Pseudomonas sp.	BS20	+	-	+	-	+	-	-
Arthrobacter sp.	BS-As2	+	-	-	-	-	+	-
Pseudomonas sp.	BS-As3	+	-	+	-	-	+	-
Pseudomonas sp.	BS-As6	+	-	-	-	-	+	-
Pseudomonas marginalis	BS-As7	-	-	+	-	+	-	-
Bacillus sp.	BS-As11	+	-	+	-	-	-	-
Brevundimonas diminuta	BS-As12	+	-	-	-	-	-	-
Pseudomonas sp.	BS-As13	+	-	+	-	-	+	-
Oerskovia sp.	BS-As14	-	-	+	-	+	-	-
Pseudomonas marginalis	BS-As19	-	+	+	-	+	-	-
Pseudomonas marginalis	BS-As22	-	+	+	-	+	+	-
Janibacter sp.	PTV7	+	-	-	-	-	-	-
Microbacterium sp.	PTV9	+	-	-	-	-	-	-
Bacillus megaterium	PTV15	+	+	-	-	-	-	-
Pedobacter sp.	PTV18	+	+	-	-	-	-	-
Brevundimonas diminuta	PTV20	+	-	+	-	-	+	-
Bacillus sp.	PTV21	+	-	-	-	-	+	-
Bacillus cereus	PTV22	+	-	-	-	-	+	-
Bacillus pumilus	PTV23	+	-	-	-	-	+	-
Janibacter sp.	PTV25	+	-	-	-	-	-	-
Brevundimonas diminuta	PTV28	+	-	-	-	-	-	-
Bacillus thuringensis	PTV30	+	+	-	-	-	-	-
Micrococcus sp.	PTV-As4	+	-	+	+	-	+	-
Brevundimonas diminuta	PTV-As5	+	-	+	-	-	+	-
Brevundimonas diminuta	PTV-As7	+	-	+	-	-	+	-
Bacillus sp.	PTV-As8	+	-	-	-	-	+	-
Janibacter sp.	PTV-As9	+	-	-	+	-	+	-
Bacillus sp.	PTV-As15	+	-	-	-	-	+	-
Bacillus pumilus	PTV-As23	+	+	-	-	+	+	-
Bacillus sp.	PTV-As26	+	-	-	-	-	+	-
Pseudomonas marginalis	PTV-As29	-	-	+	+	+	+	-

4. Discussion

Since its discovery, credited to researchers from the University of Florida, particularly Dr. Lena Q. Ma and her colleagues in the early 2000s, the Chinese brake fern *Pteris vittata* is well known for its remarkable arsenic accumulation capabilities without suffering significant damage [14,40]. Its roots have the capacity to actively absorb arsenic from the surrounding soil, transporting it to the plant's tissues through its vascular system to the fronds, which are mostly responsible for the accumulation of the metalloid (75–99%). The As-tolerance of this fern reaches 4000 ppm [14]. Moreover, this fern can be exploited in sites

characterized by multi-metal contamination due to its capability to grow in the presence of other toxic metals [41,42].

The rhizosphere, the soil region influenced by a plant's root system [43], harbors a diverse microbial community that plays a crucial role in plant health, productivity and soil ecology [44]. The characterization of the microbiota inhabiting the rhizosphere of an As-hyperaccumulating plant holds profound significance. In fact, both the epiphytic and endophytic microorganisms associated with *P. vittata* are necessarily tolerant to high metal or metalloid levels, and this specific trait can be important for host survival under stressful conditions [27]. Moreover, in phytoremediation trials, the natural occurrence or the artificial inoculation of plants with AMF can positively affect plant survival and development by mitigating the toxic effects of heavy metals and As or influencing their uptake, accumulation and translocation [27–29,31–34,45]. By harnessing the potential of rhizospheric microorganisms, *P. vittata* can efficiently thrive and hyperaccumulate arsenic and other toxic metals, thereby aiding in soil detoxification. This symbiotic association not only enhances arsenic tolerance in *P. vittata* but also amplifies its phytoremediation efficacy.

The bacterial density both in the soil and the rhizosphere of mycorrhizal *P. vittata* was quite low and did not overcome 4×10^4 CFU g^{-1} of dry soil ($\log_{10}$ CFU g^{-1} of dry soil = 4.60) in all the samples. This is consistent with the chemical analysis of the soils reported by Cantamessa et al. [24], showing multi-metal contamination in this industrial site, with As, Sb and Se levels higher than the permissible limits established by the Italian law (Legislative Decree 152/06) for industrial sites. In detail, the As level was three times the value indicated in the legislative Decree 152/06 (170 mg kg^{-1} vs. 50 mg kg^{-1}). As a direct consequence of the toxicity of these elements, the number of bacterial strains isolated, identified and characterized is not so high. High levels of arsenic in the environment may exert a strong selective pressure leading to a reduction in bacteria density and biodiversity [46], followed by the development of a few dominant bacterial species that have become well-adapted to highly arsenic-polluted soil. Moreover, no significant differences were found between the bacterial density recorded in the bulk soil and the rhizosphere, as well as in the presence or absence of As in the medium. This demonstrates that both the plant presence and As in the culture medium did not affect the culturable bacterial density.

Fourteen out of the forty-five isolated bacteria belonged to the genus *Bacillus* (31.1% of the total amount), which was the most representative, especially in the mycorrhizosphere of *P. vittata*, where its frequency reached 50%. In fact, the genus *Bacillus* is widely spread in the environment, especially in the soil, and strains belonging to this phylogenetic group frequently show As resistance mainly through arsenite methylation and oxidation [47]. In contrast, the *Pseudomonas* genus was the most representative in the bulk soil (48%) and was rarely found in the fern mycorrhizosphere. Only 1 isolate out of 20 identified as belonging to pseudomonads was found to be associated with the fern root, thus suggesting that this genus is negatively selected by the plant. This is in contrast with the literature reporting the genus *Pseudomonas* as dominant in the rhizosphere and internal root tissues of *P. vittata* [25,29,31,48]. While the role of the root exudates cannot be ruled out, the higher concentration of As around the root due to the mobilization of the metalloid by the fern may inhibit the growth of *Pseudomonas* strains, which were demonstrated to have a lower tolerance to arsenate compared to *Bacillus*. In fact, the majority (63.6%) of the Pseudomonad isolates tolerate arsenate up to 150 mM, while 50% of the strains identified as *Bacillus* tolerate arsenate up to 300 mM. The genus *Arthrobacter* was only found in the bulk soil samples. Interestingly, three out of four of the isolates belonging to this genus tolerate up to 600 mM of sodium arsenate. Only a few papers are focused on the arsenic tolerance of *Arthrobacter* strains. The MIC value for arsenate and arsenite in *Arthrobacter* sp. B6 isolated from the As-contaminated aquifer sediment in China was 150.0 mM and 5.0 mM, respectively [49]. According to Achour et al. (2017) [50], the MIC for arsenate in *Arthrobacter* sp. was 160 mM. Therefore, the arsenic tolerance shown by the strains isolated in our work is about four times that reported in the literature as typical for the genus. Moreover, all 5 isolates identified as *Brevundimonas diminuta* (4 out of 20 in the fern mycorrhizosphere and

1 out of 25 in the bulk soil) showed a very high tolerance to arsenate, reaching 600 mM. The genus *Brevundimonas* has been reported many times as being particularly resistant to As. In their work, Banerjee et al. (2021) [51] isolated the strain *Brevundimonas aurantiaca* PFAB1 from Panifala hot spring in West Bengal, India, which was tolerant to arsenite up to 90 mM and arsenate up to 310 mM. Similarly, *Brevundimonas* sp. isolated from the rhizosphere of *Oenothera picensis* plants growing close to a copper smelter in central Chile demonstrated being able to grow in high amounts of arsenic (As) (6000 mg L^{-1}) thanks to genes related to the ars operon, metal(loid)-resistance-related genes, metal efflux pumps, and detoxifying enzymes [52]. Interestingly, the *B. diminuta* strain isolated from arsenic-rich soil in the district of Uttar Pradesh (India), inoculated on rice plants exposed to 10 and 50 ppm of As, was able to increase plant biomass and plant tolerance to the metalloid through siderophore synthesis, phosphate solubilization, and auxin and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production [53].

These data are partly consistent with the literature reporting a wide range of bacterial genera collectively called arsenic-resistant bacteria (ARB), including *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium*, and *Pseudomonas*, which use As as an electron donor and acceptor or have the capability to detoxify this metalloid [47]. In addition to their role in As detoxification, these bacterial strains may directly or indirectly promote plant growth through their plant-beneficial features. About 25% of the bacterial strains isolated from the two compartments were able to synthesize IAA. This phytohormone drives the plant growth-stimulating root and xylem development, modulating the formation of lateral and adventitious roots, affecting photosynthesis and enhancing resistance to stressful conditions [54]. Siderophore producers were abundant both in the soil and the mycorrhizosphere but were more represented in the bulk soil than in the rhizosphere of mycorrhizal *P. vittata* (44% vs. 35%). It should be taken into account that in addition to their role in iron uptake, siderophores can solubilize As adsorbed on Fe-oxides, releasing As that becomes available for the plant [55,56]. An increase in the *P. vittata* biomass (+45% compared to uninoculated controls) after inoculation with bacterial strains synthesizing siderophore and IAA, coupled with an enhancement in As removal (from 13 to 35%), was reported by Lampis et al. (2015) [31].

As detoxification in arsenic-tolerant bacteria relies on the ars operons [57] encoding the influx/efflux system. There are two most common types of these operons containing either five (arsRDABC) or three (arsRBC) genes [52]. Among them, ArsC encodes an AsV reductase enzyme reducing AsV (arsenate) to AsIII (arsenite) prior to releasing it from the cell through an efflux pump encoded by arsB. ArsC enzymes belong to two unrelated families: the first one from the R773 plasmid uses glutathione and glutaredoxin as electron sources, and the second one from the pI258 plasmid is based on thioredoxin as an electron source [58]. All the bacterial strains isolated in this work showed thioredoxin-dependent arsenate reductase, while no strains possessed glutathione-dependent arsenate reductase, showing a specific selection process. Moreover, the Trx-reducing system is more efficient than the Grx-reducing system when the As concentrations are moderate or high [39]. Alternatively, AsV reduction occurs in the periplasm through a dissimilatory arsenate reductase, encoded by the arr operon, where AsV accepts a terminal electron and allows the respiration of bacteria under anaerobic conditions [59]. About 70% of the bacteria isolates selected in this work were characterized by the presence of arr genes.

It is well known that *ars* operons are widely spread in both Bacteria and Archaea species, and they have also been detected in microbes isolated from arsenic-free environments. Surprisingly, it has been stated that *ars* genes in bacteria are more common than genes for tryptophan synthesis [60], thus reflecting the ubiquitous presence of arsenic in nature. The presence of As-resistant genes on plasmids and transposons represents an opportunity for bacteria to spread these genetic traits, conferring high environmental fitness, by horizontal gene transfer. Finally, 29 out of the 45 strains (64%) showed respiratory arsenate reductase genes (arrA), considered a late evolutionary adaptation to environmental As stress [61]. All these isolates also carried ars genes. The arr operon codes

for the heterodimer respiratory arsenate reductase are made up of a large (encoded by arrA) and a small subunit (encoded by arrB) and have only been found in Bacteria and Archaea domains. Bacteria with the arr operon use arsenate as an electron acceptor and reduce it to arsenite with higher toxicity and mobility [62]. A different distribution of arrA gene variants was observed, where 48% of the strains isolated from the bulk soil showed the arrA1 gene, and 65% of the strains isolated from the mycorrhizosphere showed the arrA2 gene. A fraction corresponding to the 20% and 25% of the bacterial strains isolated from the bulk soil and the mycorrhizosphere, respectively, did not show genes involved in the respiratory arsenate reduction pathway. The different distribution of arr genes in the bulk soil and the mycorrhizosphere may be related to the different taxa identified in the two compartments.

Starting from the obtained in vitro data, it is not possible to predict the efficiency of the bacterial strains' in-plant response. Further studies are still needed to assess the ability of the different bacterial strains tested to implement the plant's ability to accumulate arsenic. However, all the tested strains, as explained above, tolerate a considerable concentration of arsenite (4 mM), and many of these grow at arsenate concentrations greater than 600 mM. We can, therefore, assume that the strains *Arthrobacter* sp. BS1 (CAS positive), *Arthrobacter* sp. BS-As2 (IAA producer), *Oerskovia* sp. BS-As14 (CAS positive), *Brevundimonas diminuta* PTV-As5 (IAA producer), *B. diminuta* PTV-As7 (IAA producer) and *Bacillus* sp. PTV-As8 (CAS positive and IAA producer) might be good candidates for further studies in plants.

In conclusion, investigating the bacterial communities associated with *Pteris vittata* in multi-metal contaminated soil can provide valuable insights for the development of more efficient phytoremediation strategies. Understanding how these bacteria interact with plants already colonized by AMF and contribute to metal accumulation and detoxification processes can enhance our ability to optimize phytoremediation efforts in polluted areas. The efficiency of this tripartite interaction (plant/bacteria/mycorrhizal fungi) can be affected by the occurrence of other pollutants, as well as other stressful conditions. In this work, we focused our attention on arsenic as the main pollutant, but other metals, such as antimony and selenium, measured over the permissible limits. It is well known that *P. vittata* can accumulate not only arsenic but also other inorganic pollutants [42,63]. This ability makes this plant species, along with its associated microbiome, an excellent tool for phytoremediation, particularly in soils contaminated with multiple metals, as in the industrial site considered in this work. The capacity of this fern to absorb various heavy metals and other harmful substances makes it an ideal choice for environmental cleanups, helping to reduce soil contamination and improve the quality of the surrounding environment. Thus, a future focus could involve exploring the possible synergies between different ecosystem components, such as the interactions between the plant, bacteria and mycorrhizal fungi, and their combined impact on the efficiency of metal uptake and detoxification. In this context, it would be important to understand how the presence of multiple contaminants influences these interactions and, consequently, the overall effectiveness of phytoremediation. Moreover, investigating the composition and function of the microbiome in the mycorrhizosphere and their role in facilitating phytoremediation could provide new insights into the mechanisms by which these microbiomes contribute to plant resistance and tolerance to pollutants, thereby offering strategies to further optimize environmental remediation technologies. Despite the importance of understanding rhizosphere microbial dynamics in phytoremediation processes, there is a notable gap in the literature concerning studies specifically targeting the rhizosphere of mycorrhizal *P. vittata*. While there are numerous studies on the plant's phytoremediation potential and its symbiotic relationship with mycorrhizal fungi, the microbial communities inhabiting the rhizosphere of mycorrhizal plants remained, till now, relatively understudied.

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