Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2024**; 29(3): 111
https://doi.org/10.31083/j.fbl2903111

Original Research

Antibacterial Properties of Bacterial Endophytes Isolated from the Medicinal Plant *Origanum heracleoticum* L.

Giulia Semenzato[1], Alberto Bernacchi[1], Sara Amata[2], Angela Bechini[3], Fabiola Berti[3], Carmela Calonico[3], Valentina Catania[4], Antonia Esposito[5], Anna Maria Puglia[6], Antonio Palumbo Piccionello[2], Giovanni Emiliani[7], Sauro Biffi[8], Renato Fani[1,*]

[1]Department of Biology, University of Florence, 50019 Sesto Fiorentino, Italy
[2]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies-STEBICEF, University of Palermo, 90128 Palermo, Italy
[3]Department of Health Sciences, University of Florence, 50134 Florence, Italy
[4]Department of Earth and Sea Science (DiSTeM), University of Palermo, 90128 Palermo, Italy
[5]Council for Agricultural and Economics Research (CREA) – Agriculture and Environment, 50125 Florence, Italy
[6]Consorzio Italbiotec, 20126 Milan, Italy
[7]Institute for Sustainable Plant Protection (IPSP)—National Research Council (CNR), 50019 Sesto Fiorentino, Italy
[8]Giardino delle Erbe Augusto Rinaldi Ceroni, 48032 Ravenna, Italy
*Correspondence: renato.fani@unifi.it (Renato Fani)

Academic Editor: Graham Pawelec
Submitted: 18 December 2023 Revised: 8 January 2024 Accepted: 16 January 2024 Published: 19 March 2024

Abstract

Background: Bacterial endophytic communities associated with medicinal plants synthesize a plethora of bioactive compounds with biological activities. Their easy isolation and growth procedures make bacterial endophytes an untapped source of novel drugs, which might help to face the problem of antimicrobial resistance. This study investigates the antagonistic potential of endophytic bacteria isolated from different compartments of the medicinal plant *O. heracleoticum* against human opportunistic pathogens. **Methods**: A panel of endophytes was employed in cross-streaking tests against multidrug-resistant human pathogens, followed by high-resolution chemical profiling using headspace-gas chromatography/mass spectrometry. **Results**: Endophytic bacteria exhibited the ability to antagonize the growth of opportunistic pathogens belonging to the *Burkholderia cepacia* complex (Bcc). The different inhibition patterns observed were related to their taxonomic attribution at the genus level; most active strains belong to the Gram-positive genera *Bacillus*, *Arthrobacter,* and *Pseudarthrobacter*. Bcc strains of clinical origin were more sensitive than environmental strains. Cross-streaking tests against other 36 human multidrug-resistant pathogens revealed the highest antimicrobial activity towards the Coagulase-negative staphylococci and *Klebsiella pneumoniae* strains. Interestingly, strains of human origin were the most inhibited, in both groups. Concerning the production of volatile organic compounds (VOCs), the strain *Arthrobacter* sp. OHL24 was the best producer of such compounds, while two *Priestia* strains were good ketones producers and so could be considered for further biotechnological applications. **Conclusions**: Overall, this study highlights the diverse antagonistic activities of *O. heracleoticum*-associated endophytes against both Bcc and multidrug-resistant (MDR) human pathogens. These findings hold important implications for investigating bacterial endophytes of medicinal plants as new sources of antimicrobial compounds.

Keywords: endophytes; medicinal plants; volatile organic compounds; antibacterial molecules

1. Introduction

The World Health Organization official definition of traditional medicine is “the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses” [1]. Medicinal plants have been used since ancient times, and traditional medicine is still practiced in a great portion of the world’s population, especially in developing countries of Africa and Asia, where these practices are perceived as more accessible and affordable, compared to modern medicine [2]. However, it is important to acknowledge that within traditional medicine, certain practices, approaches, or substances have questionable efficacy or were scientifically debunked. Among the different approaches of traditional medicine, phytotherapy has garnered a more extensive body of scientific knowledge. The curative potential of medicinal plants can be attributed to the synthesis of a wide variety of phytochemicals, which have evolved over millions of years; such compounds are involved in the interaction among organisms and in the plant response to different biotic and abiotic stresses [3,4]. Indeed, medicinal plants represent a valuable and manifold source of natural compounds with pharmaceutical potential: in many cases, the isolation and characterization of natural compounds have led to the development of widely used drugs or served as initiating steps in drug discovery [5,6].

The emergence of antimicrobial resistance, caused by the extensive and/or inappropriate use of antibiotics, has resulted in making many currently available antimicrobial drugs ineffective. The advent and spread of Multi-Drug Resistant (MDR) bacterial pathogens have become a significant and urgent public health issue, thought to put at risk 10 million lives/year in the Southeast Asian region by the year 2050 [7]. Considering the rapid worldwide dissemination of MDR clinical isolates, the discovery of new antimicrobial agents is of paramount importance [8]. Various methods have been used to obtain new effective molecules, including synthetic and combinatorial chemistry, or molecular modeling, but despite the initial interest in such synthetic techniques, the focus has recently shifted towards the employment of medicinal plants [9].

A great number of officinal plants have been recognized as a resource of antimicrobial compounds potentially effective in the treatment of MDR bacterial infections [10]. Many plants secondary metabolites, such as tannins, alkaloids, phenolic compounds, steroids, and flavonoids exhibit a growth-inhibitory action, preventing biofilm production and inhibiting bacterial virulence *in vitro* [11]; furthermore, there is evidence for the improvement of the activity of conventional antibiotics when used in combination with plant-derived compounds [12]. Hence, medicinal plants offer a very promising source of novel antibiotics in the fight against MDR bacteria: they are more available and accessible in small local communities, cheaper to purchase, easier to administrate, and have better biodegradability as compared to other available antibiotics [11]. Their structural variety is enormous and influences the antimicrobial activity they exert against different pathogenic microbes [13]. Furthermore, the effectiveness of medicinal plant extracts and essential oils (EOs) appears to be related to the synergistic effect between the multiple bioactive compounds, which lowers the chances for MDR bacteria to become resistant [14].

However, there are some challenges related to the use of plant natural products as antimicrobial pharmaceuticals, such as the lack of standardization during the preparation and storage of plant materials [15], the variation in the composition of plant extracts and EOs, which is influenced by both abiotic and biotic factors [16], or the possibility that compounds that have shown antimicrobial activity *in vitro* may have little or no effect *in vivo* [17]. Additionally, the isolation and the characterization of single compounds with the desired biological activity can be time-consuming and, in most cases, require large amounts of plant material, raising some issues regarding biodiversity conservation, as some medicinal plants are endangered and/or endemic species [18].

The genus *Origanum* represents one of the most important groups of aromatic and medicinal plants of the Lamiaceae family, distributed in warm and mountainous areas. Oregano is generally used as a spice in cooking, but it has also been employed in traditional medicine to treat respiratory disorders, stomachache, and rheumatoid arthritis, as well as a diuretic, anti-urolithic, and antimicrobial agent [19]. To date, over 100 volatile and nonvolatile compounds have been identified in the EO and extracts of *Origanum* species, the most represented being carvacrol, thymol, and rosmarinic acid [19]. *Origanum* antimicrobial efficacy is mainly exerted by its EO, making it an ideal candidate in various fields of applications, such as food preservation, natural medicine, and agricultural pest management [20]. Indeed, the EO and/or its main constituents induced remarkable inhibitory effects against MDR pathogenic bacterial strains [21–24]. However, when considering the broader *Origanum* health benefits, the synergy of various compounds, such as phenolic compounds, flavonoids, and other polyphenols, plays a crucial role [25]. The two volatile main compounds of *Origanum* EO, carvacrol and thymol, are "Generally Recognized as Safe" (ESO, GRAS-182.20) for human usage. However, the usage of those substances at the wrong concentration or for a prolonged time can induce toxic side effects to the liver, kidneys, and nervous system, and procure irritating effects to skin and mucous membranes [25].

It is widely recognized that plants' health and metabolism can be strongly influenced by the presence of specific microbial endophytes. Endophytic bacteria and/or fungi were found in nearly all vascular plant species studied: they can occupy all organs of their plant host, and some of them are seed-borne [26]. The relationship between plants and bacteria has evolved ever since plants appeared on Earth, making it possible for microbes to come up with peculiar genetic systems and metabolic pathways [27]. Little is known about how the plant determines and modulates the endophytic bacterial community composition and structure, or how endophytes influence their host, but their interaction is thought to be flexible and dynamic [28].

Medicinal plant studies mainly focused on their synthesis of bioactive phytochemicals, but recently the interest started to shift towards the exploration of their bacterial endophytic communities, as they produce a notable number of bioactive compounds that share the same or similar anticancer, anti-inflammatory, antioxidant, and antimicrobial activities [27]. Medicinal plant-associated bacterial endophytes have the potential to produce bioactive metabolites and/or modulate the plant's secondary metabolism; furthermore, some secondary metabolites may be the result of the combined metabolism of both the bacteria and their host [28,29]. Bacterial endophytes can produce different classes of bioactive compounds with biological activities, such as alkaloids, polyketones, lactones, phenolic and organic acids, flavonoids, saponins, steroids, terpenoids, phenols, peptides, and polyketides, but still represent an almost untapped source of natural molecules [27,28,30,31]. Obtaining such compounds from endophytes might offer numerous benefits: their easy isolation and growth procedures allow the preservation of the host plant, as minimal plant material is required, and the production of biologically ac-

Table 1. Endophytic bacterial strains isolated from *O. heracleoticum* used in this work.

OHF (Flowers)		OHL (Leaves)		OHS (Stems)	
Strain	Genus	Strain	Genus	Strain	Genus
1	*Bacillus*	1	*Exiguobacterium*	2	*Bacillus*
2A	*Bacillus*	2	*Bacillus*	3	*Pseudarthrobacter*
2B	*Peribacillus*	4	*Pseudarthrobacter*	4	*Bacillus*
2C	*Bacillus*	5	*Peribacillus*	5	*Bacillus*
2D	*Lysinibacillus*	6	*Exiguobacterium*	6	*Acidovorax*
2E	*Lysinibacillus*	7	*N.A.*	7	*Curtobacterium*
2G	*Bacillus*	9	*Peribacillus*	8	*Bacillus*
3	*Bacillus*	10	*Arthrobacter*	9	*Pantoea*
4	*Peribacillus*	11	*Exiguobacterium*	10	*Pseudarthrobacter*
5	*Arthrobacter*	12	*Bacillus*	11	*N.A.*
6	*Bacillus*	14	*Arthrobacter*	12	*Curtobacterium*
7	*Priestia*	15	*Neobacillus*	14	*Arthrobacter*
9	*Priestia*	16	*Labedella*	16	*Pseudomonas*
10	*Arthrobacter*	17	*Roseomonas*	18	*Pseudomonas*
11	*Bacillus*	18	*Bacillus*	19	*Erwinia*
12	*Bacillus*	20	*Bacillus*	20	*Pantoea*
13	*Cytobacillus*	21	*Peribacillus*	23	*Pseudomonas*
14	*Peribacillus*	23	*Bacillus*	24	*Pseudomonas*
15	*Pseudarthrobacter*	24	*Arthrobacter*		
16	*Bacillus*	25	*Bacillus*		
17	*Arthrobacter*				
18	*Kocuria*				
19	*Variovax*				
20	*Bacillus*				
21	*Pseudarthrobacter*				
22	*Arthrobacter*				
23	*Roseomonas*				
24	*Pseudarthrobacter*				

Abbreviations: N.A., not assigned.

tive compounds can be increased through microbial fermentation [31]. Consequently, the investigation of bacterial endophytes associated with medicinal plants may provide a solid basis for the development of novel drugs and might help to face the antimicrobial resistance issue.

Considering their potential biotechnological and pharmaceutical applications, endophytic bacterial strains were isolated from the medicinal and aromatic plant *Origanum heracleoticum* L. [32], also known as *O. vulgare* ssp. *viridulum* or *O. virens* Hoffmanns. & Link. *O. heracleoticum* is an aromatic herb widespread in the Mediterranean area. The main component of its EO is carvacrol, which is thought to be involved in its antibacterial and antifungal activity [33–35]. To the best of our knowledge, the antimicrobial potential of *O. heracleoticum*-associated bacterial endophytes has not been investigated, except for seed-associated endophytes [36]. In this study, their antibacterial activity against MDR human pathogenic strains was tested *via* cross-streaking experiments. The volatile organic compounds produced by the isolates were also characterized by headspace gas chromatography-mass spectrometry (HS/GC–MS), to shed light on the vast resource of secondary metabolites represented by the medicinal plants-associated bacterial endophytes.

2. Materials and Methods

2.1 Endophytes Growth Conditions

The bacterial strains used in this work were isolated from the endosphere of flowers, leaves, and stems of the officinal plant *O. heracleoticum*, as described in Semenzato *et al.* (2023) [32]. Strains are referred to as OH (*O. heracleoticum*), followed by the letters F, L, and S (for Flowers, Leaves, or Stems, respectively), and numbered (Table 1). The taxonomic affiliation of the bacteria was previously obtained *via* amplification and sequencing of the 16S rRNA coding gene; GenBank accession numbers are available in Semenzato *et al.* (2023) [32], except for strains OHF10 (OR880887), OHS9 (OR880888), and OHS20 (OR880889). The endophytes were stored in a 20% glycerol (453752, Carlo Erba, Milan, Italy) stock at –80 °C. They were grown on Tryptic Soy Agar (TSA, Oxoid LTD, Hampshire, UK) at 30 °C for 48 h.

2.2 Bcc Strains and Multidrug Resistant Human Pathogenic Strains Growth Conditions

Eleven strains of the *Burkholderia cepacia* complex (Bcc) belonging to four different species were selected on the basis of their origin, i.e., a clinical (Cystic Fibrosis patients, CF) or an environmental (ENV) one (Table 2). Each strain was grown on LB agar medium (NaCl 10 g/L, yeast extract 5 g/L, tryptone 10 g/L, agar 15 g/L, Oxoid LTD) at 37 °C for 48 h.

The other 36 pathogenic strains used in this work were isolated from different sources (hospital devices, foods, patients, healthy subjects, and the environment) and were previously characterized for their resistance to multiple antibiotics, using the disk-diffusion method [37]. *Staphylococcus aureus*, Coagulase-Negative Staphylococci (CoNS), *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* strains were provided by the Applied Microbiology laboratory (Health Sciences Department, University of Florence, Italy), while the standard bacteria *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603 were obtained from Thermo Fisher Diagnostics S.p.A. All the strains were grown on TSA plates at 37 °C for 24 h.

2.3 Evaluation of Antagonistic Interactions Through Cross-Streaking

Endophytes antibacterial activity against Bcc strains was evaluated through the cross-streaking method (**Supplementary Fig. 1**). Petri dishes with or without a septum separating the two compartments (to permit the growth of the tester and the target strains without any physical contact) were used. Tester strains (i.e., the *O. heracleoticum* endophytes) were streaked across one half of a TSA plate and grown at 30 °C for 48 h, to allow the synthesis of antibacterial compounds. Single colonies of each target strain were then suspended in 50 µL of a 0.9% NaCl w/v solution. Target strains belonging to the Bcc were then streaked perpendicularly to the tester strain and plates were incubated at 30 °C for a further 48 h. Additionally, Bcc strains were streaked on half of a Petri plate in the absence of the tester and were allowed to grow at 30 °C for 48 h (positive growth control). The antagonistic effect was evaluated as the reduction or absence of the target strains growth compared to control plates. The different inhibition levels were indicated as follows: complete (3), strong (2), weak (1), and absence (0) of inhibition.

Bacterial suspensions of the other MDR pathogenic strains were prepared as mentioned before and then streaked perpendicularly to the tester strain using an inoculation needle; plates were incubated at 37 °C for a further 24 h. Additionally, target strains were grown at 37 °C for 24 h in the absence of the tester (positive growth control). The antagonistic effect was evaluated as described above. The different inhibition levels were indicated as follows: complete (4), strong (3), moderated (2), weak (1), and absence of inhibition (0).

The Inhibition Score (IS) and Sensitivity Score (SS) were calculated for each tester and target strain, respectively, as the sum of the values obtained in each antagonism experiment; the total IS for each plant compartment and the total SS for CF and ENV Bcc groups, and for each MDR target group were calculated as the sum of all IS/SS, normalized by the number of tester/target strains per group (NIS and NSS). The average value of inhibition towards each MDR target group was calculated as the ratio between the sum of all IS and the number of the target strains in each group (TIS $\bar{X}$); the total average value (TOT $\bar{X}$) was calculated for each tester by dividing the total IS by the total number of MDR target strains. ANOVA was performed using R's aov() function. The degree of significance was set at $p < 0.05$. The heatmaps were obtained using the R package *pheatmap* [38].

2.4 Phylogenetic Trees

Phylogenetic trees were constructed in MEGA XI [39] by aligning, using the Muscle algorithm, the 16S rRNA gene sequences of endophytic strains belonging to the *Bacillus* and *Arthrobacter-Pseudarthrobacter* genera with 35 type strains' 16S rRNA gene sequences downloaded from the Ribosomal Database Project (RDP), selecting those exhibiting a higher percentage of identity with the endophytes sequences (≥90%) [40]. The alignment was used to build the phylogenetic trees by applying the Neighbor-Joining algorithm, with a 1000-bootstrap resampling.

2.5 Headspace-Gas Chromatography/Mass Spectrometry (HS-GC/MS) Analysis of VOCs

Biomass obtained from bacterial culture (30–100 mg) was collected in Head Space (HS) vials. Vials were sealed and immediately analyzed. HS vials were conditioned at 40 °C for 20 minutes before extraction. Bacterial volatile organic compounds (VOCs) produced by bacterial strains were extracted from the vial headspace and injected into the Gas chromatograph (GC). Headspace extraction was performed with a 2.5 mL Syringe-HS (0.64-57-R-H, PTFE, GERSTEL) conditioned and held at 40 °C from sample collection to injection. Splitless injection was used.

Gas chromatographic analysis was performed adapting a previously reported method [41]. In detail, an Agilent 7000C GC (Agilent Technologies, Inc., Santa Clara, CA, USA) system was used, equipped with a split/splitless injector, fitted with an Agilent HP5-MS UI capillary column (30 m × 250 µm; 0.25 µm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973 (Agilent Technologies, Inc., Santa Clara, CA, USA), with ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 270 °C; solvent Delay: 0 min. Helium was used as the carrier gas (1 mL/min). Concerning the oven program, the temperature was initially kept at 40 °C for 5 min, then gradually increased to 250 °C at a 2 °C/min rate; this temperature was held for 15 min and fi-

Table 2. Pathogens antimicrobial resistance profile and origin.

Genus	Strain	Antibiotic resistance	Origin
B. cepacia	FCF3		Cystic Fibrosis Patient
	LMG 1222		Environmental
B. cenocepacia	FCF23		Cystic Fibrosis Patient
	LMG 16656		Cystic Fibrosis Patient
	LMG 21462		Cystic Fibrosis Patient
	LMG 24506		Cystic Fibrosis Patient
	K56-2		Cystic Fibrosis Patient
	LMG 19230		Environmental
B. multivorans	LMG 13010		Cystic Fibrosis Patient
	LMG 17588		Environmental
B. ambifaria	LMG 19182		Environmental
CoNS	5419	FOX, DA, CIP, LEV, SXT, TIG	Food
	5318	P, E, CN, FD	Human
	5377	P, TE, E, TEC	Hospital
	5403	P, E, TIG, TE	Human
	5323	P, TE, TIG, E, CN	Human
	5321	P, E, CN, AK, FD	Human
	5284	P, TE, E, CN, FD	Hospital
	5285	E, CN, CIP, LEV, FD	Hospital
	5383	P, FOX, TE, E, CN	Hospital
	5396	P, FOX, SXT, CN, FD	Human
P. aeruginosa	ATCC 27853	FOX, K	
	5779	TOB, CAZ, FEP, MEM	Environmental
	4189	AK, TOB, CIP, LEV, CAZ, FEP, MEM, IPM, PRL, TZP	Medical device
	5234	AK, CAZ, ATM, TZP, PRL, FEP, CN, IPM, MEM, LEV, CIP, TOB	Medical device
	5245	CAZ, ATM, PRL, FEP, CN, LEV, CIP, IPM, MEM, TOB	Medical device
	7/4	CAZ, FEP, MEM, LEV, ATM	Environmental
	7/5	CAZ, FEP, MEM, TOB, ATM	Medical device
	7/3	CAZ, ATM, FEP, MEM	Environmental
	5009	ATM, CAZ, CIP, CN, FEP, IPM, LEV, MEM, PRL, TOB, TZP	Medical device
	5236	AK, ATM, CAZ, CIP, FEP, IPM, LEV, MEM, TOB	Medical device
S. aureus	ATCC 25923	P, NA	
	3428	P, C, RD, FD, TE, TGC, LZD	Medical device
	5788	P, FOX	Human
	3709	DA, TE, E	Food
	3710	DA, TE, E	Food
	4070	P, DA, TE, E, CIP, LEV, DAP	Food
	4168	AMP, P, DA, SXT, TE	Food
	4302	P, FOX, SXT, DAP	Food
	4691	P, FOX, E, CN, CIP, LEV, DAP	Hospital
	4708	P, FOX, CN, VA, DAP	Hospital
K. pneumoniae	ATCC 700603	CAZ, AMP, ATM, PRL, TE	
	4409	AK, AMX, FEP, CTX, CAZ, CIP, ETP, IPM, MEM, TZP, SXT, TIG	Human
	4412	AMX, FEP, CTX, CAZ, CIP, ETP, IPM, MEM, TZP, SXT, TIG	Human
	4417	AK, AMX, FEP, CTX, CAZ, CIP, ETP, IPM, MEM, TZP, SXT, TIG	Human
	4420	AK, AMX, FEP, CTX, CAZ, CIP, IPM, MEM, TZP, SXT, CN	Human
	4422	AK, AMX, FEP, CTX, CAZ, CIP, ETP, IPM, MEM, TZP, SXT	Human

Abbreviations: AK, Amikacin; AMX, Amoxicillin; AMP, Ampicillin; ATM, Aztreonam; CTX, Cefotaxime; CAZ, Ceftazidime; CIP, Ciprofloxacin; CN, Gentamicin; DA, Clindamycin; DAP, Daptomycin; ETP, Ertapenem; E, Erythromycin; FD, Fusidic acid; FEP, Cefepime; FOX, Cefoxitin; IPM, Imipenem; K, Kanamycin; LEV, Levofloxacin; MEM, Meropenem; NA, Nalidixic acid; P, Penicillin G; PRL, Piperacillin; SXT, Sulfamethoxazole/trimethoprim; TE, Tetracycline; TEC, Teicoplanin; TIG, Tigecycline; TOB, Tobramycin; TZP, Piperacillin/tazobactam; VA, Vancomycin.

nally raised to 270 °C at a 10 °C/min rate. Samples were injected at 250 °C automatically. Interval scan: 35–450 m/z; Scan speed: 10,000 amu·s^{-1} (25 Hz). The GC–MS mass spectrum data were analyzed using MassHunter Qualitative Analysis B.06.00 and the database of the National Institute Standard and Technology (NIST) was used to interpret the analyzed data. A comparison between the mass spectrum of the unidentified components released by the bacterial isolates and the mass spectrum of already known components available in the NIST 11 MS library was carried out (Match factor >800).

3. Results and Discussion

3.1 Cross-Streaking with and without Septum against Bcc strains

We first checked the ability of the entire panel of bacterial endophytes to inhibit the growth of eleven bacterial strains belonging to the Bcc, bacterial strains known for their resistance to numerous conventional antibiotics. Bcc species are responsible for infections in Cystic Fibrosis (CF) patients, leading to severe health complications such as necrotizing pneumonia and sepsis, reducing patients' survival rates [42]. Treatment of Bcc infections is challenging because of their intrinsic resistance to various antibiotics and their tolerance to antibiotic exposure, especially in biofilm formations [43]. Current therapeutic approaches lack evidence-based guidelines and often follow various antibiotic protocols. However, the complete eradication of Bcc infection remains difficult, prompting the exploration of alternative strategies, such as the identification of compounds able to enhance antibiotic activity by targeting resistance mechanisms, or the employment of alternative therapies such as quorum-sensing inhibitors, natural antimicrobial peptides, and specifically designed vaccines [44].

Data obtained are shown in Fig. 1, whose analysis revealed that almost all the endophytes (with few exceptions, see for instance *Peribacillus* sp. OHF10 and *Arthrobacter* sp. OHS14) were able to inhibit the growth of at least two Bcc strains, even though at a different extent: some of them were able to completely interfere with the growth of the entire panel of Bcc strains (*Bacillus* sp. OHF9, *Cytobacillus* sp. OHF13, and *Pseudarthrobacter* sp. OHS10), while others exhibited a moderate/low degree of inhibition. In general, *O. heracleoticum*-associated strains were much more able to reduce or inhibit the growth of Bcc strains isolated from CF patients (NSS = 115) than those with an environmental origin (NSS = 72). This was previously observed for medicinal plants-associated bacteria isolated from different plant compartments and rhizospheric soil of *Origanum vulgare* L., *Lavandula angustifolia* Mill., and *Echinacea purpurea* L. [45–47]. The most sensitive strain was FCF3 (SS = 153), while LMG1222 (SS = 56) was the most resistant. Although the two strains belong to the same species (*B. cepacia*), their behavior in response to the antagonistic activity of the endophytes was completely different, suggesting the relevant role of the strains origin in determining their resistance profiles [48]. This finding is quite interesting, and it is worth further investigation. In our opinion, CF Bcc isolates obtained from healthcare settings may have been exposed to specific antibiotics or antimicrobial agents, potentially influencing their resistance profiles. In contrast, ENV strains might face a different selective pressure, including exposure to various stressors and competition, which could influence their differential response to antibacterial compounds [49]. Over time, these strains may have developed mechanisms to survive in the presence of antimicrobial agents found in their natural habitat, leading to higher resistance levels compared to clinical isolates.

The analysis of the NIS revealed that the flowers compartment hosted the community with the highest inhibitory potential against the Bcc strains, with a NIS of 21, compared to the leaves and stems compartments (NIS = 17). Concerning the single strains, the highest IS (from 33 to 26) were registered for strains belonging to the genera *Arthrobacter* (OHF5, OHF22, OHL24), *Pseudarthrobacter* (OHF15, OHF21, OHF24, OHS3, OHS10), *Bacillus* (OHL23, OHS8), *Cytobacillus* (OHF13), *Peribacillus* (OHF2B), *Priestia* (OHF7, OHF9), *Pantoea* (OHS9), and *Curtobacterium* (OHS12). It is worth noting that the majority of the most active strains are Gram-positive, belonging to the Phyla Firmicutes and Actinobacteria, except for *Pantoea* sp. OHS9. It is widely recognized that the phylum Actinobacteria is responsible for over half of the natural bioactive compounds documented in literature surveys, encompassing antibiotics, immunosuppressive agents, antitumor agents, and enzymes [50]. They can be isolated from various environmental sources, such as soil samples, plants, and the marine environment, and in many cases, their potential to attenuate and/or inhibit the growth of Gram-negative and Gram-positive human pathogenic strains was documented [51,52]. Antibacterial activities were also reported for the Phylum Firmicutes, especially for isolates belonging to the genus *Bacillus* [53,54]. A previous work on bacterial endophytes isolated from *O. vulgare* L. reported similar results; indeed, the most active endophytic strains were all Gram-positive (with few exceptions), many of which belonged to the genera *Arthrobacter* and *Bacillus* [45]. Consistently, the most active bacterial endophytes associated with *E. purpurea* rhizospheric soil were affiliated to the genus *Arthrobacter* [47]. From these first observations, we can hypothesize that the different antibacterial patterns observed for *O. heracleoticum*-associated bacterial strains seem not related to the ecological niche from which they were isolated but might be linked to their taxonomical affiliation at the genus level.

The same results are also reported in Fig. 2. In this case, the tester and target strains were sorted based on hierarchical clustering (dendrograms on the top and the left of the heatmap), highlighting well-defined groups of strains.

				TARGET STRAINS											
				CF							ENV				
				B. cepacia	*B. cenocepacia*	*B. multivorans*	*B. cenocepacia*	*B. cenocepacia*	*B. cenocepacia*	*B. cenocepacia*	*B. cepacia*	*B. multivorans*	*B. ambifaria*	*B. cenocepacia*	Inhibition score
				FCF3	FCF23	LMG13010	LMG16656	LMG21462	LMG24506	K56-2	LMG1222	LMG17588	LMG19182	LMG19230	
TESTER STRAINS	FLOWERS	*Bacillus*	OHF1	3	1	0	2	3	3	2	1	1	2	1	19
		Bacillus	OHF2A	3	3	1	3	3	3	3	1	1	2	1	24
		Peribacillus	OHF2B	3	3	1	3	3	3	3	1	2	2	2	26
		Bacillus	OHF2C	3	2	1	2	3	3	2	1	1	2	1	21
		Bacillus	OHF3	3	2	1	3	3	3	3	1	1	2	0	22
		Peribacillus	OHF4	1	1	1	1	1	1	1	1	0	0	0	8
		Arthrobacter	OHF5	3	3	2	3	3	3	3	2	2	2	2	28
		Bacillus	OHF6	2	0	0	0	2	0	0	0	0	0	0	4
		Priestia	OHF7	3	3	2	3	3	3	3	2	2	3	2	29
		Priestia	OHF9	3	3	3	3	3	3	3	3	3	3	3	33
		Peribacillus	OHF10	0	0	0	0	0	0	0	0	0	0	0	0
		Bacillus	OHF11	3	1	0	1	2	2	1	0	0	1	0	11
		Cytobacillus	OHF13	3	3	3	3	3	3	3	3	3	3	3	33
		Peribacillus	OHF14	3	2	1	2	3	2	2	1	1	1	1	19
		Pseudarthrobacter	OHF15	3	3	2	3	3	3	3	2	2	3	2	29
		Bacillus	OHF16	3	2	0	2	3	3	3	1	0	2	1	20
		Arthrobacter	OHF17	3	2	1	2	3	3	2	1	2	2	2	23
		Kocuria	OHF18	3	2	0	2	3	2	2	0	1	2	1	18
		Variovorax	OHF19	3	2	1	2	2	2	1	0	1	0	1	15
		Bacillus	OHF20	2	0	0	1	1	1	0	0	0	1	0	6
		Pseudarthrobacter	OHF21	3	3	2	3	3	3	3	2	2	3	3	30
		Arthrobacter	OHF22	3	3	1	3	3	3	3	2	2	2	2	27
		Roseomonas	OHF23	3	1	0	2	3	2	2	1	0	1	1	16
		Pseudarthrobacter	OHF24	3	3	3	3	3	3	3	2	3	3	3	32
															Flowers normalized inhibition score	**21**
	LEAVES	*Exiguobacterium*	OHL1	2	1	0	1	2	1	1	0	1	0	0	9
		Bacillus	OHL2	3	2	1	2	3	3	2	2	1	2	1	22
		Pseudarthrobacter	OHL4	3	3	1	2	3	3	2	1	2	2	2	24
		Peribacillus	OHL5	3	1	1	1	2	2	1	0	0	1	0	12
		Exiguobacterium	OHL6	2	1	0	1	3	1	0	0	0	1	0	9
		N.A.	OHL7	2	1	0	1	2	1	1	0	0	0	0	8
		Peribacillus	OHL9	3	1	0	2	2	2	1	1	0	1	1	14
		Arthrobacter	OHL10	3	3	1	2	3	3	2	1	2	2	2	24
		Exiguobacterium	OHL11	2	1	0	1	3	2	1	0	0	1	0	11
		Bacillus	OHL12	3	2	1	2	3	3	2	1	1	1	1	20
		Arthrobacter	OHL14	3	1	0	2	3	2	1	0	1	1	0	14
		Neobacillus	OHL15	3	2	1	1	3	3	2	1	1	1	3	21
		Labedella	OHL16	3	1	0	1	3	2	2	0	0	1	0	13
		Roseomonas	OHL17	2	0	0	1	1	1	0	0	0	0	0	5
		Bacillus	OHL18	3	1	0	1	3	2	1	0	0	1	1	13
		Peribacillus	OHL21	3	2	1	2	3	3	2	1	1	1	1	20
		Bacillus	OHL23	3	3	3	3	3	3	3	2	3	3	1	30
		Arthrobacter	OHL24	3	3	3	3	3	3	3	2	3	3	3	32
															Leaves normalized inhibition score	**17**
	STEMS	*Bacillus*	OHS2	1	0	0	0	1	0	0	1	0	1	0	4
		Pseudarthrobacter	OHS3	3	3	2	3	3	2	2	3	2	3	2	28
		Bacillus	OHS4	1	0	0	1	1	1	0	0	1	2	0	7
		Bacillus	OHS5	3	3	1	2	3	2	2	0	2	2	0	20
		Acidovorax	OHS6	3	3	2	2	2	2	2	0	2	2	2	22
		Curtobacterium	OHS7	2	0	0	1	2	1	0	0	1	1	0	8
		Bacillus	OHS8	3	3	2	3	3	2	2	3	2	3	2	28
		Pantoea	OHS9	3	3	2	3	3	2	3	1	3	3	3	29
		Pseudarthrobacter	OHS10	3	3	3	3	3	3	3	3	3	3	3	33
		N.A.	OHS11	2	1	0	0	2	2	0	0	0	1	0	8
		Curtobacterium	OHS12	3	3	3	3	3	3	2	0	3	3	3	29
		Arthrobacter	OHS14	0	0	0	0	0	0	0	0	0	0	0	0
		Pseudomonas	OHS16	2	3	2	2	2	2	2	1	2	2	2	22
		Pseudomonas	OHS18	1	2	0	1	1	1	0	2	0	1	0	9
		Erwinia	OHS19	3	2	1	1	3	2	1	0	2	2	1	18
		Pantoea	OHS20	3	3	1	3	3	3	3	1	1	1	1	23
		Pseudomonas	OHS23	2	0	0	0	1	1	0	0	0	0	0	4
		Pseudomonas	OHS24	1	0	0	1	1	0	0	1	0	1	0	5
															Stems normalized inhibition score	**17**
			Sensitivity score	153	109	58	110	147	126	100	56	70	95	67		
			Total normalized sensitivity score	115							72					

Fig. 1. Cross streaking against Burkholderia cepacia complex (Bcc) strains using plates without septum. Different inhibition levels were indicated as follows: complete (3, red), strong (2, orange), weak (1, yellow), and absence of inhibition (0, white). Inhibition and sensitivity scores were calculated by adding all the values obtained for each tester or target strain, respectively. CF, cystic fibrosis patient; ENV, environmental origin.

Target strains (columns) were divided into three clusters: the first one, on the left, groups the most sensitive strains, all of clinical origin (*B. cepacia* FCF3, *B. cenocepacia* LMG21465, and LMG506); the middle one includes three CF strains and one ENV strain (*B. ambifaria* LMG19182), which were strongly or completely inhibited by around 60% of tester endophytic strains; finally, the last group (on the right of the heatmap) is mostly composed of ENV strains, except for *B. multivorans* LMG13010, and include the most resistant Bcc strains. Hence, the hierarchical clustering confirmed that environmental strains are generally more resistant than clinical isolates ($p = 0.02$, ANOVA test).

Concerning the endophytes' clustering (rows), the first group is formed by the most active tester strains, already listed in the previous paragraph; the second group consists of strains that were able to strongly or completely inhibit Bcc strains included in the first two target clusters;

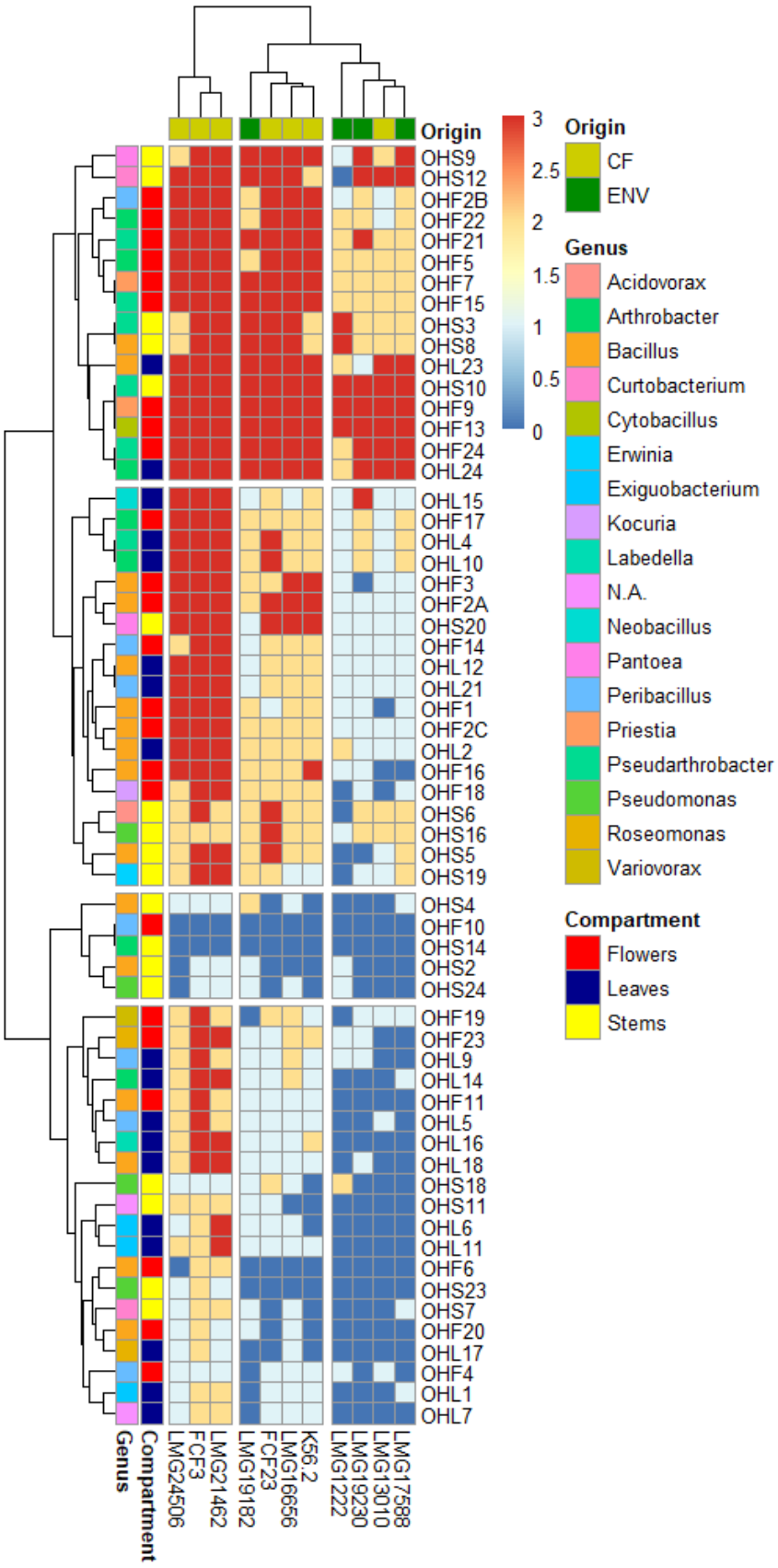

Fig. 2. Heatmap representation of the different inhibitory activity of O. heracleoticum endophytic strains against the Bcc group (without septum). Rows represent the different endophytic testers, while columns are the target strains. Cells color is based on the observed reduction or inhibition of target growth. Both columns and rows are ordered based on hierarchical clustering (dendrograms).

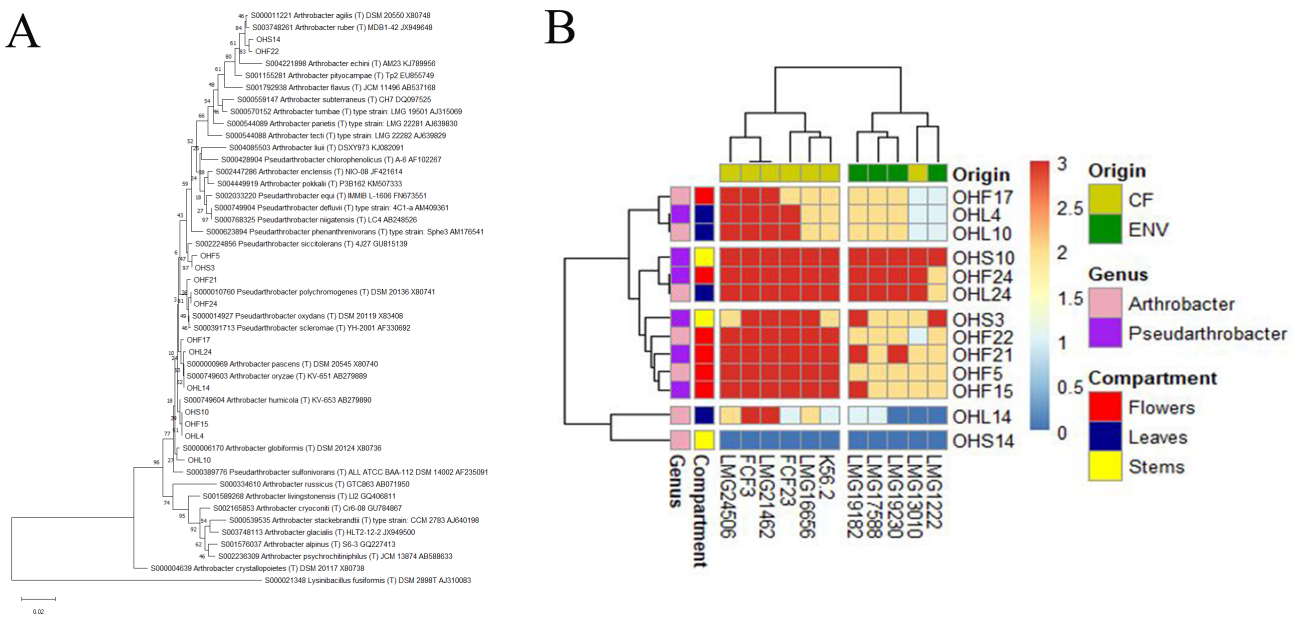

Fig. 3. Phylogeny and antagonistic interactions within the genera *Arthrobacter* and *Pseudarthrobacter*. (A) Phylogenetic tree for the genera *Arthrobacter* and *Pseudarthrobacter*. (B) Heatmap representation of the different inhibitory activity of endophytic strains belonging to genera *Arthrobacter* and *Pseudarthrobacter* against the Bcc group (without septum). Rows represent the different testers, while columns are the target strains. Cells color is based on the observed reduction or inhibition of target growth. Both columns and rows are ordered based on hierarchical clustering (dendrograms).

the third one groups five endophytes with very low or no antagonistic effect on Bcc strains; lastly, the fourth group includes strains with low IS.

For each endophyte, the taxonomic affiliation at the genus level and the compartment of origin were color-coded on the left of the heatmap. 56.3% of endophytic strains belonging to the first group were isolated from the flowers compartment (9 out of 16 strains), while only two strains (12.5%) belonged to the leaves-associated endophytic community. The nine OHF strains belong to the genera *Arthrobacter*, *Pseudarthrobacter*, *Bacillus*, *Cytobacillus*, *Peribacillus*, and *Priestia*, the latter four all belonging to the family Bacillaceae. On the other hand, 4 out of 5 endophytes (80%) of the third group were associated with the stems compartment and belonged to the genera *Pseudomonas*, *Bacillus*, and *Arthrobacter*. From these first observations, we can hypothesize that the different antibacterial patterns observed for *O. heracleoticum*-associated bacterial strains seem not related to the ecological niche from which they were isolated but might be linked to their phylogenetic relatedness. For example, *Pseudarthrobacter* sp. OHF24 and OHS10, isolated from flowers and stems, respectively, have similar activity against Bcc target strains and belong to the same species [32]. Indeed, the ANOVA test (**Supplementary Table 1**) confirmed that the IS obtained for each endophytic strain was significantly related to the taxonomic affiliation of the strain at the genus level ($p < 0.05$), but not to the compartment from which they were isolated. A non-significant interaction between the two factors was also observed. Thus, taxonomic affiliation is apparently involved in determining endophytes antagonistic activity against Bcc strains, but it cannot be excluded an interplay between taxonomy and ecological niche; indeed, it has been demonstrated that the composition of the bacterial communities living inside plants' different compartments is not casual, and this might determine different antagonistic patterns in different anatomical parts [47,55].

To ascertain which of these two parameters, i.e., the "ecological niche" or "taxonomic affiliation", is strongly associated with the ability of endophytes to inhibit Bcc strains, our attention focused on the two main phylogenetic subgroups: the first is composed by 13 strains belonging to the genera *Arthrobacter* and *Pseudarthrobacter*, while the second one includes 16 strains belonging to the genus *Bacillus*. For each of the two subgroups, a phylogenetic tree based on 16S rRNA gene sequences and a heatmap portraying cross-streaking results against Bcc strains were obtained (Figs. 3 and 4).

Both subgroups include strains with (very) dissimilar inhibition patterns isolated from different plant organs. Concerning the *Arthrobacter* subgroup heatmap (Fig. 3B), tester strains, sorted based on hierarchical clustering, were divided into 5 clusters of strains, formed by (i) OHS10, OHF24, and OHL24, which exhibited the strongest antibacterial activity towards both CF and ENV Bcc strains; (ii) OHS3, OHF22, OHF21, OHF5, and OHF15, which were slightly less efficient against ENV Bcc strains; (iii) OHF17, OHL4, and OHL10, with reduced anti-Bcc activity compared to the previous two groups; (iv) OHL14 strain, which was able to interfere with the growth of

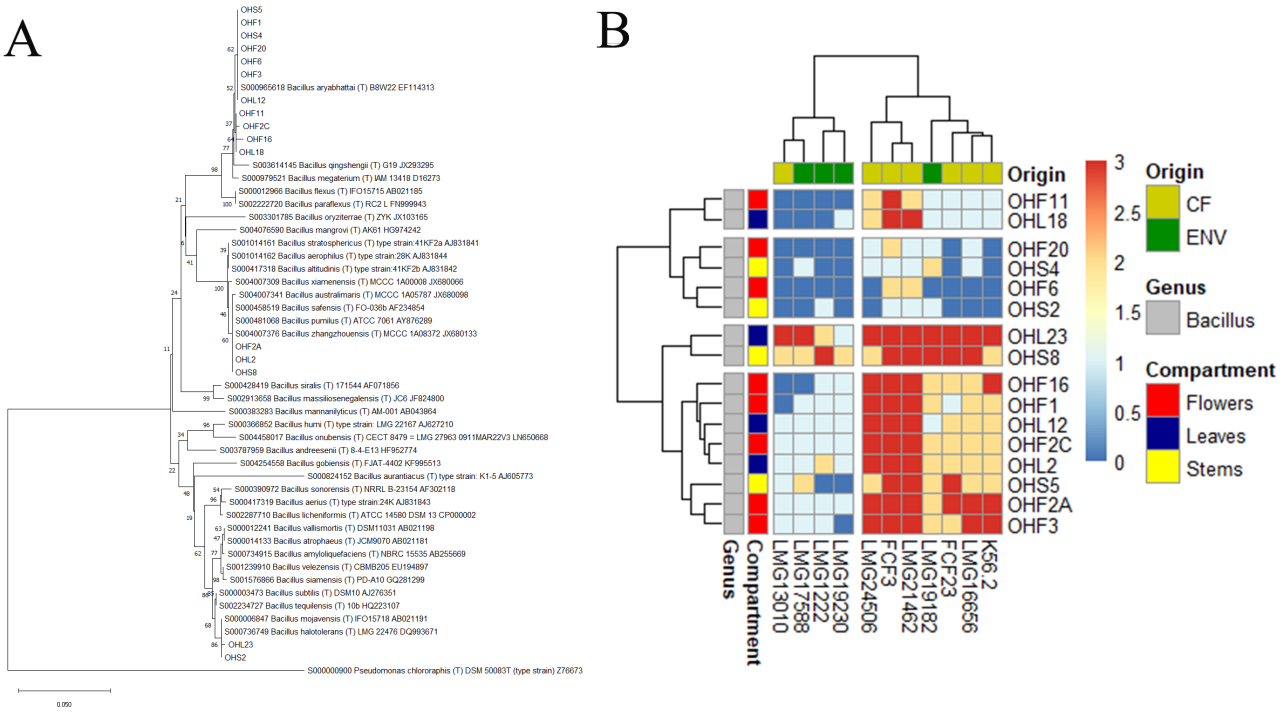

Fig. 4. Phylogeny and antagonistic interactions within the genus *Bacillus*. (A) Phylogenetic tree for the genus *Bacillus*. (B) Heatmap representation of the different inhibitory activity of endophytic strains belonging to the genus *Bacillus* against the Bcc group (without septum). Rows represent the different testers, while columns are the target strains. Cells color is based on the observed reduction or inhibition of target growth. Both columns and rows are ordered based on hierarchical clustering (dendrograms).

Burkholderia strains FCF3, LMG21462, LMG24506, and LMG16656 only; (v) OHS14, with no antibacterial activity. Concerning the *Bacillus* subgroup (Fig. 4B), the 4 clusters are composed of (i) OHS8 and OHL23, which strongly or completely inhibited the growth of all target strains; (ii) OHF16, OHF1, OHL12, OHF2C, OHL2, OHS5, OHF2A, and OHF3, which were active only towards clinical target strains; (iii) OHF11 and OHS18, whose antagonistic effect was limited to *Burkholderia* strains FCF3, LMG21462, and LMG24506; (iv) OHF20, OHS4, OHF6, and OHS2, which have no or low antagonistic effect.

As can be seen from the annotation on the left of the heatmaps, there is no evident clustering of strains based on the compartment of origin ($p > 0.05$, ANOVA test), except for the *Arthrobacter* cluster II, where 4 out of 5 strains were isolated from the flowers compartment. Observing the *Arthrobacter* and *Pseudarthrobacter* phylogenetic tree (Fig. 3A), only a few strain clusters are evident. One of them is formed by the endophytic strains OHF5 and OHS3: they belong to the same Operational Taxonomic Unit (OTU, percentage of identity = 99%) and both obtained an IS of 28, indeed they are grouped in the same section of the heatmap. However, on the other hand, strains OHS14 and OHF22 belong to the same OTU (percentage of identity = 99%) but have completely different inhibitory activities against Bcc strains (IS_{OHF22} = 27; IS_{OHS14} = 0). A similar pattern can be found also in the *Bacillus* subgroup. Strains OHL23 and OHS2 belong to the same OTU (percentage of identity = 99%), but the obtained IS for the two strains are divergent (IS_{OHL23} = 30; IS_{OHS2} = 4). Nevertheless, *Bacillus* strains OHL18 and OHL11, which are the only strains included in the heatmap cluster III, are in the same OTU (percentage of identity = 99%), and the same can be observed for strains OHF6, OHF20 and OHS4 (IS ranging from 4 to 7 and all belonging to cluster IV of the heatmap), which, however, share the same OTU with OHL12, OHF3, OHS5, and OHF1, strains with higher IS (ranging from 19 to 22 and all belonging to cluster II of the heatmap). In general, the inhibition pattern varied between strains probably belonging to the same species, suggesting that antibacterial activity might be due to different inhibitory mechanisms within the same species and so be related to intra-specific genomic differences.

The observed antagonistic activity of endophytes might be attributed to the production of diffusible but also volatile molecules with antibacterial activity. Since volatile organic compounds (VOCs) are involved not only in inter/intra-species communication but also in antagonism between microbes [56], the production of VOCs was analyzed on a smaller subset of 12 endophytes, all clustered in the first group of the heatmap (Fig. 2), isolated from all three compartments, and belonging to the genera *Arthrobacter* (OHF5, OHF24, and OHL24), *Pseudarthrobacter* (OHF15, OHF21, OHS3, and OHS10), *Bacillus* (OHL23 and OHS8),

			TARGET STRAINS											
			CF							ENV				
			B.cepacia	*B. cenocepacia*	*B. multivorans*	*B. cenocepcia*	*B. cenocepcia*	*B. cenocepcia*	*B. cenocepcia*	*B. cepacia*	*B. multivorans*	*B. ambifaria*	*B. cenocepcia*	**Inhibition score**
			FCF3	FCF23	LMG13010	LMG16656	LMG21462	LMG24506	K56-2	LMG1222	LMG17588	LMG19182	LMG19230	
TESTER STRAINS	*Arthrobacter*	OHF5	3	3	2	3	3	3	3	2	2	3	2	29
	Priestia	OHF7	2	3	0	3	3	3	2	1	2	1	0	20
	Priestia	OHF9	3	2	0	3	3	3	3	1	2	2	0	22
	Pseudarthrobacter	OHF15	3	3	2	3	3	3	3	2	2	2	1	27
	Pseudarthrobacter	OHF21	1	3	3	3	3	3	3	2	3	3	2	29
	Arthrobacter	OHF24	3	3	3	3	3	3	3	2	2	3	2	30
	Bacillus	OHL23	3	3	2	3	3	3	3	2	2	3	2	29
	Arthrobacter	OHL24	3	3	2	3	3	3	3	2	3	3	2	30
	Pseudarthrobacter	OHS3	3	3	2	3	3	3	3	2	2	3	1	28
	Bacillus	OHS8	3	3	3	3	3	3	3	2	3	3	2	31
	Pseudarthrobacter	OHS10	2	3	2	3	3	3	3	2	2	3	1	27
	Curtobacterium	OHS12	2	0	0	0	0	0	0	0	0	0	0	2
Sensitivity score			31	32	21	33	33	33	32	20	25	29	15	

Fig. 5. Cross streaking against Bcc strains using plates with septum. Different inhibition levels were indicated as follows: complete (3, red), strong (2, orange), weak (1, yellow), and absence of inhibition (0, white). Inhibition and sensitivity scores were calculated as the sum of all the values obtained for each tester or target strain, respectively.

Priestia (OHF7 and OHF9) and *Curtobacterium* (OHS12). The cross-streaking tests were performed on Petri dishes divided into two halves by a physical septum, not allowing the diffusion of antibacterial molecules from the tester to the target strains. The obtained results are reported in Fig. 5.

All the selected strains, apart from *Curtobacterium* sp. OHS12, were able to strongly or completely antagonize the growth of CF and ENV Bcc strains. Due to the presence of the physical septum, the antibacterial activity of these endophytes could be attributed solely to the production of VOCs. Also in this case, the antibacterial effect was stronger towards CF targets but still substantial against ENV Bcc strains. Overall, the inhibition patterns and the IS obtained in this experiment were the same or slightly lower than the ones obtained using plates without septum. However, three strains exhibit interesting discrepancies with respect to the IS shown in the cross-streaking experiments performed with Petri dishes without a septum. The two *Priestia* strains (OHF7 and OHF9), exhibiting an IS of 29 and 33, respectively, in the previous experiments, showed a decreased IS, which is particularly marked for Bcc strains of environmental origin. The third strain, *Curtobacterium* sp. OHS12, did not affect the growth of Bcc strains through the production of VOCs.

The most active strains, scoring an IS of 30, were *Arthrobacter* sp. OHF24 and OHL24, isolated respectively from the flowers and the leaves compartment and not close phylogenetically (Fig. 3A). In a previous work, the antibacterial potential of VOCs produced by a panel of 8 endophytic strains isolated from *O. vulgare* was evaluated against 10 Bcc strains [41]. Also in this case, the most active strain, OVS8, belonged to the genus *Arthrobacter*, which was also able to interfere with the growth of human MDR *Klebsiella pneumoniae* strains through the produc-

			TARGET STRAINS																																				Inhibition score
			CoNS										*K. Pneumoniae*						*S. aureus*										*P. aeruginosa*										
TESTER STRAINS			5323	5321	5318	5403	5377	5396	5284	5285	5383	5419	4420	4412	4417	4409	4422	700603	4691	5788	3428	4302	3710	4168	4070	3709	4708	25923	5236	4189	5245	07\05	5009	07\04	27853	5234	07\03	5779	
FLOWERS	*Arthrobacter*	OHF5	3	3	3	3	3	3	1	1	1	0	3	3	3	3	2	2	2	2	2	2	2	1	1	1	2	1	1	1	0	1	0	2	0	0	1	0	59
FLOWERS	*Cytobacillus*	OHF13	2	2	1	2	2	0	2	0	1	0	3	2	2	3	2	3	0	0	1	1	1	1	1	1	0	1	0	0	2	0	1	0	0	0	0	0	37
FLOWERS	*Priestia*	OHF7	1	1	1	0	2	1	1	2	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	17
FLOWERS	*Priestia*	OHF9	2	1	2	2	2	0	2	3	0	0	0	2	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19
FLOWERS	*Pseudarthrobacter*	OHF15	3	3	3	2	2	1	1	1	1	0	3	3	3	3	2	2	2	1	2	1	1	1	1	1	1	1	2	2	2	1	1	0	0	0	1	0	54
FLOWERS	*Pseudarthrobacter*	OHF21	3	3	3	3	3	3	2	2	2	0	3	3	3	3	2	2	2	3	1	0	1	2	1	1	2	1	1	2	1	2	1	1	1	1	1	1	66
FLOWERS	*Pseudarthrobacter*	OHF24	3	3	3	3	2	2	1	1	1	0	3	3	3	3	2	2	2	2	1	1	1	1	2	2	2	1	2	1	2	1	1	1	1	1	0	0	60
	Flowers normalized inhibition score																																						**45**
LEAVES	*Arthrobacter*	OHL24	3	3	3	3	3	2	2	1	1	0	3	2	3	3	2	1	1	1	1	1	1	0	0	0	1	1	2	2	1	1	1	2	2	2	0	1	56
LEAVES	*Bacillus*	OHL23	3	3	3	3	3	3	2	3	3	2	1	0	0	0	0	0	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0	59
	Leaves normalized inhibition score																																						**58**
STEMS	*Bacillus*	OHS8	4	4	4	4	2	4	4	4	4	4	3	2	3	1	3	3	2	2	2	2	2	2	2	2	2	2	2	2	0	2	1	0	0	0	0	0	80
STEMS	*Pantoea*	OHS9	1	1	1	3	1	1	3	0	0	0	3	1	0	1	0	1	1	1	2	1	1	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	27
STEMS	*Curtobacterium*	OHS12	3	3	3	2	2	2	1	0	1	0	2	1	2	2	2	0	1	1	0	2	1	0	0	0	0	0	1	1	2	0	0	0	0	1	0	0	36
STEMS	*Pseudarthrobacter*	OHS3	3	3	3	3	3	3	2	2	1	0	2	4	2	2	0	1	2	1	1	2	1	2	2	1	1	0	2	2	1	1	1	0	1	0	1	1	57
STEMS	*Pseudarthrobacter*	OHS10	3	3	3	3	3	2	1	1	1	0	3	3	3	3	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	0	0	1	1	0	0	50
	Stems normalized inhibition score																																						**50**
Sensitivity score			37	36	36	36	33	27	25	21	17	6	33	30	28	28	19	17	20	19	18	18	17	15	15	15	15	12	14	14	13	11	7	6	6	6	4	3	
Total normalized sensitivity score			27										26						16										8										

Fig. 6. Cross streaking against multidrug resistant (MDR) pathogenic strains using plates without septum. Different inhibition levels were indicated as follows: complete (4, deep red), strong (3, red), moderate (2, orange), weak (1, yellow), and absence of inhibition (0, white). Inhibition and sensitivity scores were calculated by adding all the values obtained for each tester or target strain, respectively.

tion of VOCs [57]. Within the Actinomycetes phylum, research studies mainly focused on the genus *Streptomyces*, which is widely known for its remarkable ability to produce a wide array of antibiotics and other bioactive compounds and thus employed in various medical, agricultural, and industrial applications [58]. Indeed, there is still limited evidence regarding the antibacterial compounds synthesized by *Arthrobacter* species, with most studies focusing, to date, on strains isolated from Antarctic or marine environments [51,59,60]. The results here obtained suggest the potentiality of *Arthrobacter* strains isolated from medicinal plants as a promising source of antibacterial molecules of diffusible and volatile nature.

3.2 Cross-Streaking with and without Septum against Multidrug Resistant Human Pathogens

The endophytic strains showing the highest inhibitory activity against Bcc strains were also tested against 36 MDR human pathogens, belonging to *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and CoNS groups. The strains were selected because of their resistance to multiple antibiotics (Table 2). Moreover, the interest in these species is validated by their inclusion in the AR-ISS program in Italy, which actively participates in the European Antimicrobial Resistance Surveillance Network (EARS-Net), overseen by the European Centre for Disease Prevention and Control (ECDC). The primary goal of AR-ISS surveillance is to describe the antibiotic resistance within a specific group of pathogens isolated from invasive infections belonging to eight species (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* species), to provide insights into the current status of antibiotic resistance and contribute in global efforts to address this growing concern in healthcare [61]. The same pathogenic species were previously employed in cross-streaking experiments aimed at demonstrating the antibacterial potential of *O. vulgare* bacterial endophytes; some of them, belonging to the genera *Bacillus* and *Arthrobacter*, were mostly active towards CoNS and *S. aureus* strains [45].

The antibacterial activity of the target strains was evaluated qualitatively, as described in the previous paragraphs. Data shown in Fig. 6 revealed that the selected endophytes reported the highest antimicrobial activity against the CoNS group (except for strain 5419, isolated from a food sample), with a NSS = 27, followed by *K. pneumoniae* (NSS = 26) and *S. aureus* (NSS = 16) groups. On the contrary, *P. aeruginosa* strains were the most resistant (NSS = 8). Interestingly, the most inhibited strains belonging to the CoNS group are the ones of human origin, followed by strains isolated from hospital devices. This is quite relevant considering the potential applications of the 12 endophytic strains as antibiotics producers able to face the spread of MDR human pathogens. As verified by the ANOVA analysis, MDR target strains' sensitivity was significantly related to both taxonomic affiliation and isolation source ($p < 0.001$).

Contrary to what was observed for the Bcc group, the flowers compartment hosted the community with the lower inhibitory potential, with a NIS of 45, compared to the leaves (NIS = 58) and stems compartments (NIS = 50). The highest IS was reported for the tester strain *Bacillus* sp. OHS8 (IS = 80), which was able to completely inhibit the growth of almost all the CoNS strains and induced a

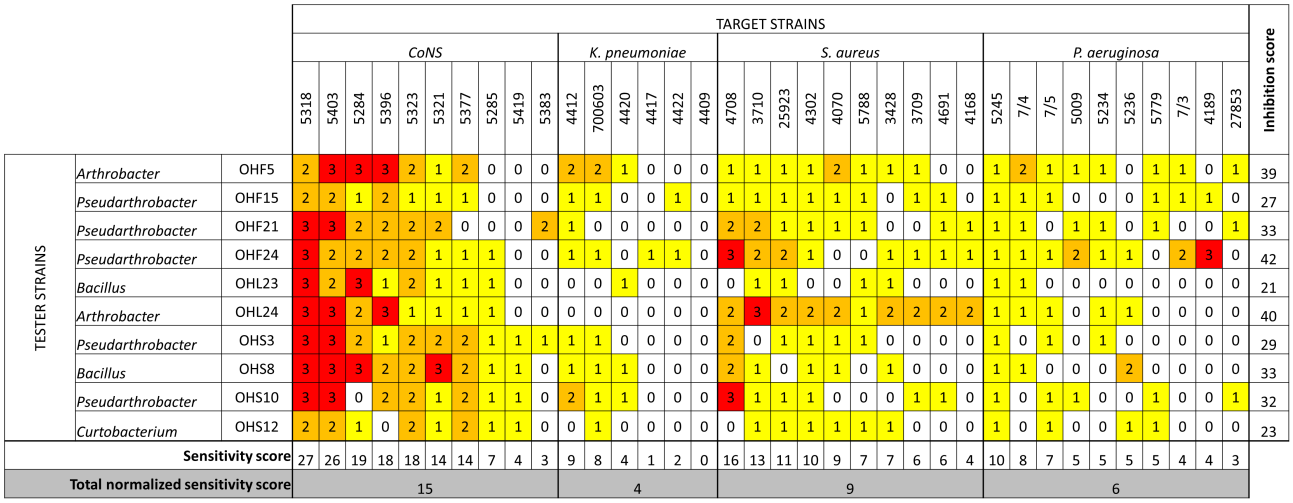

Fig. 7. Cross streaking against MDR pathogenic strains using plates with septum. Different inhibition levels were indicated as follows: complete (4, deep red), strong (3, red), moderate (2, orange), weak (1, yellow), and absence of inhibition (0, white). Inhibition and sensitivity scores were calculated by adding all the values obtained for each tester or target strain, respectively.

Table 3. Cross-streaking without septum results expressed as the total inhibition score (TIS) for each target group, the average value of TIS for each target group ($\bar{X}$), and the total value of TIS and $\bar{X}$ (TOT) for each tester.

Genus	Strain	CoNS		*K. pneumoniae*		*P. aeruginosa*		*S. aureus*		TOT	TOT
		TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$
Arthrobacter	OHF5	21	2.1	16	2.7	16	1.6	6	0.6	59	1.7
Cytobacillus	OHF13	12	1.2	15	2.5	7	0.7	3	0.3	37	1.2
Priestia	OHF7	9	0.9	5	0.8	2	0.2	1	0.1	17	0.5
Priestia	OHF9	14	1.4	2	0.3	3	0.3	0	0.0	19	0.5
Pseudarthrobacter	OHF15	17	1.7	16	2.7	12	1.2	9	0.9	54	1.6
Pseudarthrobacter	OHF21	24	2.4	16	2.7	14	1.4	12	1.2	66	1.9
Pseudarthrobacter	OHF24	19	1.9	16	2.7	15	1.5	10	1.0	60	1.8
Arthrobacter	OHL24	21	2.1	14	2.3	7	0.7	14	1.4	56	1.6
Bacillus	OHL23	28	2.8	1	0.2	30	3.0	0	0.0	59	1.5
Bacillus	OHS8	38	3.8	15	2.5	20	2.0	7	0.7	80	2.3
Pantoea	OHS9	11	1.1	6	1.0	10	1.0	0	0.0	27	0.8
Curtobacterium	OHS12	17	1.7	9	1.5	5	0.5	5	0.5	36	1.1
Pseudarthrobacter	OHS3	23	2.3	11	1.8	13	1.3	10	1.0	57	1.6
Pseudarthrobacter	OHS10	20	2.0	13	2.2	10	1.0	7	0.7	50	1.5

strong-moderate inhibition against *K. pneumoniae* and *S. aureus* groups. Apart from OHS8, the highest IS (from 56 to 66) were registered for strains belonging to the genera *Pseudarthrobacter* (OHF21, OHF24, OHS3), *Arthrobacter* (OHF5, OHL24), and *Bacillus* (OHL23). In particular, *Bacillus* sp. OHL23 was the only endophyte able to strongly inhibit the growth of all the *S. aureus* strains. On the other hand, the lowest antibacterial activity was reported for the two *Priestia* strains (OHF7 and OHF9), which had almost no antagonistic effect on *P. aeruginosa* and *S. aureus* groups.

Based on these results, 10 endophytic strains were tested for their ability to produce VOCs capable of antagonizing the growth of the 36 MDR human pathogenic strains, using Petri dishes with a septum physically separating the plate into two compartments, as described in the Materials and Methods section. Data obtained are shown in Fig. 7.

In general, *O. heracleoticum* endophytes antibacterial activity was lower compared to the previous cross-streaking test results. Tester strains VOCs exhibited a moderate antagonistic effect towards the CoNS strains (NSS = 15), especially against *S. epidermidis* 5318 and 5403, both of human origin. The highest IS, ranging from 42 to 39, were registered for strains belonging to the genera *Arthrobacter* (OHF5, OHL24) and *Pseudarthrobacter* (OHF24).

Concerning the cross streaking performed against MDR pathogenic strains, the total inhibition score (TIS) and the average value of TIS for each target group ($\bar{X}$) and the total average value for each tester (TOT $\bar{X}$) were calculated (Tables 3 and 4). The tester strains reported the highest

Table 4. Cross-streaking with septum results expressed as the total inhibition score (TIS) for each target group, the average value of TIS for each target group ($\bar{X}$), and the total value of TIS and $\bar{X}$ (TOT) for each tester.

Genus	Strain	CoNS		*K. pneumoniae*		*P. aeruginosa*		*S. aureus*		TOT	TOT
		TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$	TIS	$\bar{X}$
Arthrobacter	OHF5	16	1.6	5	0.8	9	0.9	9	0.9	39	1.1
Pseudarthrobacter	OHF15	10	1.0	3	0.5	6	0.6	8	0.8	27	0.7
Pseudarthrobacter	OHF21	16	1.6	1	0.2	6	0.6	10	1.0	33	0.9
Pseudarthrobacter	OHF24	14	1.4	4	0.7	12	1.2	12	1.2	42	1.1
Bacillus	OHL23	14	1.4	1	0.2	2	0.2	4	0.4	21	0.6
Arthrobacter	OHL24	15	1.5	0	0.0	5	0.5	20	2.0	40	1.0
Pseudarthrobacter	OHS3	18	1.8	2	0.3	3	0.3	6	0.6	29	0.8
Bacillus	OHS8	20	2.0	3	0.5	4	0.4	6	0.6	33	0.9
Pseudarthrobacter	OHS10	15	1.5	4	0.7	5	0.5	8	0.8	32	0.9
Curtobacterium	OHS12	12	1.2	1	0.2	4	0.4	6	0.6	23	0.6

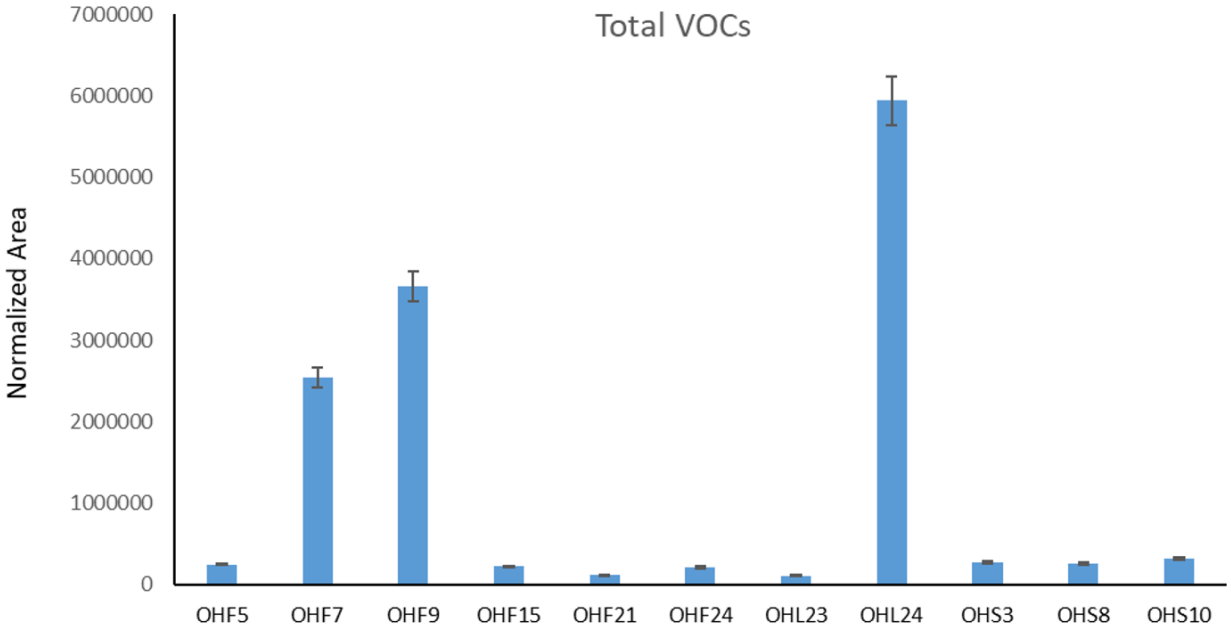

Fig. 8. Total Volatile organic compounds (VOCs) produced by each strain. Histogram bars represent the total normalized area of the chromatogram peaks.

antimicrobial activity against the CoNS group in both experiments (the $\bar{X}$ value ranged from 0.9 to 3.8 without septum; from 0.2 to 2.8 with septum), while *S. aureus* strains in cross-streaking experiments without septum ($\bar{X}$ value from 0.0 to 1.4), and the *K. pneumoniae* group when using Petri dishes with a septum ($\bar{X}$ value from 0.0 to 0.8) were only slightly inhibited.

3.3 Identification of VOCs Employing HS-GC/MS

The VOCs produced by the most interesting strains were analyzed using HS-GC/MS. The identified VOCs are listed in Table 5. Results are expressed as the area of each peak in the chromatograms normalized by the bacterial biomass. Headspace-GC (HS-GC/MS) was chosen since it reduces sample manipulation, does not require the use of solvents, and because of its easy preparative steps [41]. A total of 16 distinct metabolites were detected, mainly belonging to the following structurally distinct classes: alcohols (1-Butanol, 1-Butanol-3-methyl, 1-Hexanol, 2-ethyl-, 2-Propanol), ketones (2-Butanone, 2-Butanone, 3-methyl-, Acetone), hemiterpenes (isoprene), and sulphurated compounds (Bis(methylthio) methane, Carbon disulfide, Dimethyl sulfide, Dimethyl disulfide, Dimethyl trisulfide, Ethanethioic acid S-methyl ester, Metanthiol, Thiophene) (see Table 5).

Among all strains, *Arthrobacter* sp. OHL24 exhibited the highest VOCs content based on the normalized area of all peaks, followed by *Priestia* strains OHF9 and OHF7 (Fig. 8).

Table 5. Volatile organic compounds (VOCs) identified by HS-GC/MS produced by bacterial strains. Results are expressed as mean relative abundance percentages (as obtained by dividing the normalized area of each peak by the total area of the chromatogram peaks).

	1-Butanol	1-Butanol-3-methyl	1-Hexanol, 2-ethyl-	2-Propanol	2-Butanone	2-Butanone, 3-methyl-	Acetone	Isoprene	Bis(methylthio) methane	Carbon disulfide	Dimethyl sulfide	Dimethyl disulfide	Dimethyl trisulfide	Ethanethioic acid, S-methyl ester	Metanthiol	Thiophene
Arthrobacter sp. OHF5	4.33	7.07	18.36	13.74	3.91	2.83	6.92	5.04	0.00	2.29	13.20	17.32	0.29	0.00	0.00	4.68
Priestia sp. OHF7	2.05	0.00	0.00	0.90	1.39	0.10	94.70	0.14	0.00	0.07	0.31	0.26	0.00	0.00	0.00	0.09
Priestia sp. OHF9	1.78	0.92	0.00	1.94	1.12	0.09	93.27	0.13	0.00	0.08	0.31	0.24	0.00	0.00	0.00	0.10
Pseudarthrobacter sp. OHF15	3.92	6.63	13.20	23.26	4.09	1.69	7.85	5.33	0.00	1.32	17.12	12.58	0.17	0.00	0.00	2.86
Pseudarthrobacter sp. OHF21	5.26	6.51	16.22	23.27	6.45	2.15	7.32	2.87	0.00	1.61	15.27	9.83	0.25	0.00	0.00	3.00
Pseudarthrobacter sp. OHF24	4.94	7.35	17.00	18.96	5.96	3.70	10.71	4.63	0.00	0.76	13.84	7.78	0.23	0.00	0.00	4.14
Bacillus sp. OHL23	6.05	7.51	20.66	12.28	4.37	1.92	9.17	5.88	0.00	2.35	15.24	9.67	0.20	0.00	0.00	4.69
Arthrobacter sp. OHL24	0.36	0.30	7.58	0.00	0.78	0.19	0.63	0.29	0.05	0.72	35.59	51.41	0.11	0.14	1.72	0.15
Pseudarthrobacter sp. OHS3	7.45	6.04	25.77	14.41	6.85	2.63	12.99	3.18	0.00	1.08	8.62	7.13	0.23	0.00	0.00	3.63
Bacillus sp. OHS8	3.57	1.84	8.11	20.56	1.85	1.27	31.88	2.57	0.01	0.81	18.67	5.87	0.15	0.00	0.00	2.84
Pseudarthrobacter sp. OHS10	8.07	14.51	15.44	25.99	4.91	1.69	7.84	2.41	0.00	1.13	10.61	4.63	0.10	0.00	0.00	2.68

Based on the relative peak area, the compound dimethyl disulfide was the most abundant volatile for *Arthrobacter* sp. OHL24. Among sulfides, dimethyl sulfide was produced by almost all strains. It was demonstrated that dimethyl disulfide induced significant growth inhibition of different microbial pathogens, such as *Rhizoctonia solani* and *Pythium ultimum*, or many Gram-negative and Gram-positive bacteria [62].

For *Pseudarthrobacter* strains OHF15, OHF21, OHF24, and OHS10, 2-propanol was the most abundant compound, while it was acetone for *Priestia* sp. OHF7 and sp. OHF9, and *Bacillus* sp. OHS8. Notably, *Priestia* strains OHF7 and OHF9 produced acetone as the main compound (>93% total VOCs). 1-Hexanol-2-ethyl was the main component for *Arthrobacter* sp. OHF5, *Bacillus* sp. OHL23 and *Pseudarthrobacter* sp. OHS3. The distribution of the different classes of metabolites among all strains is shown in **Supplementary Figs. 2–5**.

The general antibacterial activity of the endophytic strains can be correlated to the production of thiophene, while the higher activity of *Arthrobacter* sp. OHL24 against *S. aureus* strains could be ascribed to the production of sulphurated compounds.

4. Conclusions

Overall, the present study highlighted that endophytic bacteria associated with the medicinal plant *O. heracleoticum* exhibited, although to a very different extent, the ability to antagonize the growth of the human opportunistic pathogens belonging to the *B. cepacia* complex. The different inhibition patterns observed are not related to the plant compartment from which the endophytes were isolated, but to their taxonomic classification at the genus level; this assumption has been confirmed with a deeper analysis at the species level focused on two of the most represented and most active bacterial genera, *Bacillus* and the *Arthrobacter/Pseudarthrobacter* group. Bcc strains showed different sensitivity to the endophytes, which was attributed to their origin (clinical strains are more sensitive than environmental strains). Cross-streaking tests against the 36 MDR human pathogens revealed the highest antimicrobial activity towards the CoNS and *K. pneumoniae* strains. Consistently, the strains of human origin were the most inhibited, in both target groups. On the contrary, *P. aeruginosa* strains isolated from the environment or medical devices showed the lowest susceptibility. Cross-streaking tests using plates with a septum confirmed the strains' ability to produce VOCs with antimicrobial properties, able to induce a strong antagonist effect towards the Bcc strains and a moderate one against the CoNS strains of human origin. Sulphurated compounds were responsible for the prominent antibacterial activity of *Arthrobacter* sp. OHL24 against *S. aureus* strains. Moreover, strains *Priestia* sp. OHF7 and OHF9 were good ketones producers and could be considered for further biotechnological applications. Further tests to investigate non-diffusible metabolites with antibacterial activity are in due course. In conclusion, this study points up the diverse antagonistic capabilities of *O. heracleoticum*-associated endophytes against both Bcc and MDR human pathogens, shedding light on taxonomic and biochemical factors contributing to their antimicrobial activities. These findings hold important implications for investigating new sources of antibacterial compounds and comprehending the intricate relationships that exist between medicinal plants and endophytic bacteria.

Availability of Data and Materials

The 16S rRNA gene sequences of the endophytic strains used in this work are available in GenBank (NCBI) under the accession numbers from ON979588 to ON979656, OR880887, OR880888, and OR880889.

Author Contributions

RF and GS designed the research study. GS, AE, ABer, FB, VC, SA, and APP performed the experiments. CC, ABec, SB, AMP, APP, GE, and RF supervised the experimental work. SB provided the initial plant material. GS, FB, CC, and APP wrote the original draft. GS cured the visualization of data. ABer, SA, ABec, VC, AE, AMP, GE, SB, and RF reviewed and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

Given their role as Guest Editors of the journal, Renato Fani, Giovanni Emiliani, and Giulia Semenzato had no involvement in the peer-review of this article and have no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Graham Pawelec. All other authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2903111.

References

[1] WHO. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. World Health Organization: Geneva. 2000.

[2] Oyebode O, Kandala NB, Chilton PJ, Lilford RJ. Use of traditional medicine in middle-income countries: a WHO-SAGE study. Health Policy and Planning. 2016; 31: 984–991.

[3] Guerriero G, Berni R, Muñoz-Sanchez JA, Apone F, Abdel-Salam EM, Qahtan AA, *et al*. Production of Plant Secondary Metabolites: Examples, Tips and Suggestions for Biotechnologists. Genes. 2018; 9: 309.

[4] Süntar I. Importance of ethnopharmacological studies in drug discovery: role of medicinal plants. Phytochemistry Reviews. 2020; 19: 1199–1209.

[5] Salmerón-Manzano E, Garrido-Cardenas JA, Manzano-Agugliaro F. Worldwide Research Trends on Medicinal Plants. International Journal of Environmental Research and Public Health. 2020; 17: 3376.

[6] McChesney JD, Venkataraman SK, Henri JT. Plant natural products: back to the future or into extinction? Phytochemistry. 2007; 68: 2015–2022.

[7] Coque TM, Graham DW, Pruden A, So AD, Topp E, Vaughn Grooters S, *et al*. United Nations Environment Programme, Bracing for Superbugs. Strengthening environmental action in the One Health response to antimicrobial resistance. 2023. Available at: https://www.unep.org/resources/superbugs/environmental-action (Accessed: 20 November 2023).

[8] Manandhar S, Luitel S, Dahal RK. *In Vitro* Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria. Journal of Tropical Medicine. 2019; 2019: 1895340.

[9] Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. Life Sciences. 2005; 78: 431–441.

[10] Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery. Metabolites. 2019; 9: 258.

[11] Vaou N, Stavropoulou E, Voidarou C, Tsigalou C, Bezirtzoglou E. Towards Advances in Medicinal Plant Antimicrobial Activity: A Review Study on Challenges and Future Perspectives. Microorganisms. 2021; 9: 2041.

[12] Phitaktim S, Chomnawang M, Sirichaiwetchakoon K, Dunkhunthod B, Hobbs G, Eumkeb G. Synergism and the mechanism of action of the combination of α-mangostin isolated from Garcinia mangostana L. and oxacillin against an oxacillin-resistant Staphylococcus saprophyticus. BMC Microbiology. 2016; 16: 195.

[13] Qadri H, Haseeb Shah A, Mudasir Ahmad S, Alshehri B, Almilaibary A, Ahmad Mir M. Natural products and their semi-synthetic derivatives against antimicrobial-resistant human pathogenic bacteria and fungi. Saudi Journal of Biological Sciences. 2022; 29: 103376.

[14] Lopes TS, Fontoura PS, Oliveira A, Rizzo FA, Silveira S, Streck AF. Use of plant extracts and essential oils in the control of bovine mastitis. Research in Veterinary Science. 2020; 131: 186–193.

[15] Ong ES. Extraction methods and chemical standardization of botanicals and herbal preparations. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 2004; 812: 23–33.

[16] Sadeh D, Nitzan N, Chaimovitsh D, Shachter A, Ghanim M, Dudai N. Interactive effects of genotype, seasonality and extraction method on chemical compositions and yield of essential oil from rosemary (Rosmarinus officinalis L.). Industrial Crops and Products. 2019; 138: 111419.

[17] Tsao R, Li H. Antioxidant properties *in vitro* and *in vivo*: Realistic assessments of efficacy of plant extracts. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 2012; 7.

[18] Alvin A, Miller KI, Neilan BA. Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiological Research. 2014; 169: 483–495.

[19] Soltani S, Shakeri A, Iranshahi M, Boozari M. A Review of the Phytochemistry and Antimicrobial Properties of *Origanum vulgare* L. and Subspecies. Iranian Journal of Pharmaceutical Research: IJPR. 2021; 20: 268–285.

[20] Lombrea A, Antal D, Ardelean F, Avram S, Pavel IZ, Vlaia L, *et al*. A Recent Insight Regarding the Phytochemistry and Bioactivity of *Origanum vulgare* L. Essential Oil. International Journal of Molecular Sciences. 2020; 21: 9653.

[21] Coccimiglio J, Alipour M, Jiang ZH, Gottardo C, Suntres Z. Antioxidant, Antibacterial, and Cytotoxic Activities of the Ethanolic Origanum vulgare Extract and Its Major Constituents. Oxidative Medicine and Cellular Longevity. 2016; 2016: 1404505.

[22] Pesavento G, Maggini V, Maida I, Lo Nostro A, Calonico C, Sassoli C, *et al*. Essential Oil from Origanum vulgare Completely Inhibits the Growth of Multidrug-Resistant Cystic Fibrosis Pathogens. Natural Product Communications. 2016; 11: 861–864.

[23] Rosato A, Vitali C, Piarulli M, Mazzotta M, Argentieri MP, Mallamaci R. *In vitro* synergic efficacy of the combination of Nystatin with the essential oils of Origanum vulgare and Pelargonium graveolens against some Candida species. Phytomedicine: International Journal of Phytotherapy and Phytopharmacology. 2009; 16: 972–975.

[24] De Martino L, De Feo V, Formisano C, Mignola E, Senatore F. Chemical composition and antimicrobial activity of the essential oils from three chemotypes of Origanum vulgare L. ssp. hirtum (Link) Ietswaart growing wild in Campania (Southern Italy). Molecules (Basel, Switzerland). 2009; 14: 2735–2746.

[25] Martins N, Barros L, Santos-Buelga C, Henriques M, Silva S, Ferreira ICFR. Decoction, infusion and hydroalcoholic extract of Origanum vulgare L.: different performances regarding bioactivity and phenolic compounds. Food Chemistry. 2014; 158: 73–80.

[26] Tosi M, Gaiero J, Linton N, Mafa-Attoye T, Castillo A, Dunfield K. Bacterial Endophytes: Diversity, Functional Importance, and Potential for Manipulation. Rhizosphere Biology: Interactions Between Microbes and Plants. 2021; 155–168.

[27] Tanvir R, Javeed A, Bajwa AG. Endophyte bioprospecting in South Asian medicinal plants: an attractive resource for biopharmaceuticals. Applied Microbiology and Biotechnology. 2017; 101: 1831–1844.

[28] Wu W, Chen W, Liu S, Wu J, Zhu Y, Qin L, *et al*. Beneficial Relationships Between Endophytic Bacteria and Medicinal Plants. Frontiers in Plant Science. 2021; 12: 646146.

[29] Yadav G, Meena M. Bioprospecting of endophytes in medicinal plants of Thar Desert: An attractive resource for biopharmaceuticals. Biotechnology Reports (Amsterdam, Netherlands). 2021; 30: e00629.

[30] Webster G, Mullins AJ, Cunningham-Oakes E, Renganathan A, Aswathanarayan JB, Mahenthiralingam E, *et al*. Culturable diversity of bacterial endophytes associated with medicinal plants of the Western Ghats, India. FEMS Microbiology Ecology. 2020; 96: fiaa147.

[31] Makuwa SC, Serepa-Dlamini MH. The Antibacterial Activity of Crude Extracts of Secondary Metabolites from Bacterial Endophytes Associated with *Dicoma anomala*. International Journal of Microbiology. 2021; 2021: 8812043.

[32] Semenzato G, Del Duca S, Vassallo A, Zaccaroni M, Mucci N, Greco C, *et al*. Exploring the nexus between the composition of essential oil and the bacterial phytobiome associated with different compartments of the medicinal plants Origanum vulgare ssp. vulgare, O. vulgare ssp. hirtum, and O. heracleoticum. Industrial

Crops and Products. 2023; 191: 115997.
[33] Della Pepa T, Elshafie HS, Capasso R, De Feo V, Camele I, Nazzaro F, *et al*. Antimicrobial and Phytotoxic Activity of *Origanum heracleoticum* and *O. majorana* Essential Oils Growing in Cilento (Southern Italy). Molecules (Basel, Switzerland). 2019; 24: 2576.
[34] Salgueiro LR, Cavaleiro C, Pinto E, Pina-Vaz C, Rodrigues AG, Palmeira A, *et al*. Chemical composition and antifungal activity of the essential oil of Origanum virens on Candida species. Planta Medica. 2003; 69: 871–874.
[35] Zengin G, Cvetanović A, Gašić U, Dragićević M, Stupar A, Uysal A, *et al*. UHPLC-LTQ OrbiTrap MS analysis and biological properties of Origanum vulgare subsp. viridulum obtained by different extraction methods. Industrial Crops and Products. 2020; 154: 112747.
[36] Semenzato G, Faddetta T, Falsini S, Del Duca S, Esposito A, Padula A, *et al*. Endophytic Bacteria Associated with Origanum heracleoticum L. (Lamiaceae) Seeds. Microorganisms. 2022; 10: 2086.
[37] Delfino V, Calonico C, Nostro AL, Castronovo LM, Duca SD, Chioccioli S, *et al*. Antibacterial activity of bacteria isolated from *Phragmites australis* against multidrug-resistant human pathogens. Future Microbiology. 2021; 16: 291–303.
[38] Kolde R. pheatmap: Pretty Heatmaps. Available at: https://CRAN.R-project.org/package=pheatmap (Accessed: 21 November 2023).
[39] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution. 2018; 35: 1547–1549.
[40] Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, *et al*. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research. 2014; 42: D633–42.
[41] Polito G, Semenzato G, Del Duca S, Castronovo LM, Vassallo A, Chioccioli S, *et al*. Endophytic Bacteria and Essential Oil from *Origanum vulgare* ssp. *vulgare* Share Some VOCs with an Antibacterial Activity. Microorganisms. 2022; 10: 1424.
[42] Drevinek P, Mahenthiralingam E. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases. 2010; 16: 821–830.
[43] Lord R, Jones AM, Horsley A. Antibiotic treatment for Burkholderia cepacia complex in people with cystic fibrosis experiencing a pulmonary exacerbation. The Cochrane Database of Systematic Reviews. 2020; 4: CD009529.
[44] Leitão JH, Sousa SA, Ferreira AS, Ramos CG, Silva IN, Moreira LM. Pathogenicity, virulence factors, and strategies to fight against Burkholderia cepacia complex pathogens and related species. Applied Microbiology and Biotechnology. 2010; 87: 31–40.
[45] Castronovo LM, Calonico C, Ascrizzi R, Del Duca S, Delfino V, Chioccioli S, *et al*. The Cultivable Bacterial Microbiota Associated to the Medicinal Plant *Origanum vulgare* L.: From Antibiotic Resistance to Growth-Inhibitory Properties. Frontiers in Microbiology. 2020; 11: 862.
[46] Emiliani G, Mengoni A, Maida I, Perrin E, Chiellini C, Fondi M, *et al*. Linking Bacterial Endophytic Communities to Essential Oils: Clues from Lavandula angustifolia Mill. Evidence-based Complementary and Alternative Medicine: ECAM. 2014; 2014: 650905.
[47] Chiellini C, Maida I, Maggini V, Bosi E, Mocali S, Emiliani G, *et al*. Preliminary data on antibacterial activity of Echinacea purpurea-associated bacterial communities against Burkholderia cepacia complex strains, opportunistic pathogens of Cystic Fibrosis patients. Microbiological Research. 2017; 196: 34–43.
[48] Araque-Calderon Y, Miranda-Contreras L, Rodriguez-Lemoine V, Palacios-Pru EL. Antibiotic resistance patterns and SDS-PAGE protein profiles of Burkholderia cepacia complex isolates from nosocomial and environmental sources in Venezuela. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 2008; 14: BR49–BR55.
[49] Lupo A, Coyne S, Berendonk TU. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Frontiers in Microbiology. 2012; 3: 18.
[50] Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Meier-Kolthoff JP, *et al*. Taxonomy, Physiology, and Natural Products of Actinobacteria. Microbiology and Molecular Biology Reviews: MMBR. 2015; 80: 1–43.
[51] Claverías FP, Undabarrena A, González M, Seeger M, Cámara B. Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaíso bay, Chile. Frontiers in Microbiology. 2015; 6: 737.
[52] Elbendary AA, Hessain AM, El-Hariri MD, Seida AA, Moussa IM, Mubarak AS, *et al*. Isolation of antimicrobial producing *Actinobacteria* from soil samples. Saudi Journal of Biological Sciences. 2018; 25: 44–46.
[53] Ismail A, Ktari L, Ahmed M, Bolhuis H, Boudabbous A, Stal LJ, *et al*. Antimicrobial Activities of Bacteria Associated with the Brown Alga Padina pavonica. Frontiers in Microbiology. 2016; 7: 1072.
[54] Yilmaz M, Soran H, Beyatli Y. Antimicrobial activities of some Bacillus spp. strains isolated from the soil. Microbiological Research. 2006; 161: 127–131.
[55] Maida I, Chiellini C, Mengoni A, Bosi E, Firenzuoli F, Fondi M, *et al*. Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant Echinacea purpurea. Environmental Microbiology. 2016; 18: 2357–2365.
[56] Veselova MA, Plyuta VA, Khmel IA. Volatile Compounds of Bacterial Origin: Structure, Biosynthesis, and Biological Activity. Microbiology (Russian Federation). Pleiades Publishing. 2019; 88: 261–274.
[57] Semenzato G, Del Duca S, Vassallo A, Bechini A, Calonico C, Delfino V, *et al*. Genomic, Molecular, and Phenotypic Characterization of *Arthrobacter* sp. OVS8, an Endophytic Bacterium Isolated from and Contributing to the Bioactive Compound Content of the Essential Oil of the Medicinal Plant *Origanum vulgare* L. International Journal of Molecular Sciences. 2023; 24: 4845.
[58] Donald L, Pipite A, Subramani R, Owen J, Keyzers RA, Taufa T. Streptomyces: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. Microbiology Research. 2022; 13: 418–465.
[59] Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice A, Michaud L, *et al*. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against Burkholderia cepacia complex bacteria. Biotechnology Advances. 2012; 30: 272–293.
[60] Undabarrena A, Beltrametti F, Claverías FP, González M, Moore ERB, Seeger M, *et al*. Exploring the Diversity and Antimicrobial Potential of Marine Actinobacteria from the Comau Fjord in Northern Patagonia, Chile. Frontiers in Microbiology. 2016; 7: 1135.
[61] Iacchini S, Boros S, Pezzotti P, Caramia A, Errico G, Del Grosso M, *et al*. AR-ISS: sorveglianza nazionale dell'Antibiotico-Resistenza. 2022. Available at: https://www.iss.it/ (Accessed: 20 December 2023).
[62] Ossowicki A, Jafra S, Garbeva P. The antimicrobial volatile power of the rhizospheric isolate Pseudomonas donghuensis P482. PLoS ONE. 2017; 12: e0174362.