Antibiotic and heavy metal resistant endophytes inhabit Armeria maritima hyperaccumulator

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Research Article

Keywords: Antibiotic resistance, Armeria maritima, Metallophytes, Endophytes, Phytoremediation, Pseudomonas

Posted Date: April 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2845134/v1

Abstract

Background and Aims

The occurrence of heavy metal-resistant bacteria in plants and their role in phytoremediation intensification has been quite well recognized in the last few years. Nevertheless, there is a dearth of information on antibiotic resistance profile of those bacteria. In this study, for the first time endophytic bacteria has been isolated from green parts of *Armeria maritima* sp. halleri plant growing on mine-tailing soil in the southern Poland. The resistance profile of bacteria was researched.

Methods

Bacteria were isolated from internal tissues of *Armeria maritima* plant and characterized: MIC was determined by the plate dilution method using $(CH_3COO)_2Pb$ and $ZnSO_4$ supplemented medium; antibiotic susceptibility test was determined by disk diffusion method according to the EUCAST version 11.0; the whole genome sequencing was performed on MiSeq platform (Illumina). Physicochemical properties of soil were evaluated according to European Standards.

Results

Five *Pseudomonas* sp. strains exhibiting high tolerance to heavy metals were resistant to antibiotics, such as aminoglycosides, ß-lactam antibiotics, fosfomycines fluorochinolones, macrolides and glycopeptides. Draft genome sequences analysis estimated genome sizes in a range from 6,182,403bp to 7,401,235bp and a G + C content from 60.0–61.0%. 12 and 11 genes conferring resistance heavy metals and antibiotics were identified, respectively.

Conclusion

Armeria maritima subsp. halleri is inhabited by resistant to heavy metals and antibiotic endophytic bacteria identified as *Pseudomonas* species. Under the One Health concept the contamination of soil and plants with ARB and ARGs should be monitored and limited and a regulatory framework for safety use of bacterial bioinoculants should be established.

Introduction

Careless management of agricultural and industrial activities have derived into serious contamination of soils with heavy metals. It poses significant risk to public health so new biosafety and effective technologies reducing this contamination are needed. A commonly used technology to remove metals from soil is phytoextraction. This is a kind of phytoremediation method which involves hyperaccumulating plants use to decrease heavy metal level in contaminated areas (Kumar et al. 1995). Hyperaccumulators are capable of concentrating extremely high levels of heavy metals in their tissues. An example of such plant is *Armeria maritima* spp. halleri. According to many authors *Armeria maritima* species can be divided in three ecotypes, such as *A. maritima* subsp. maritima growing on salt marsh, *A. maritima* subsp. elongate growing on dry sandy soils and *A. maritima* subsp. halleri inhabiting metalliferrous soils (Purmale et al. 2022). *Armeria maritima* subsp. halleri is able to remove metal ions through salt glands formatted on the leaf epidermis, it can transport metal ions from cytoplasm to shoot's cell wall, it accumulates metal ions in the root apoplast and it can bind the metal ions with special ligands in the cytoplasm (Olko et al. 2008). Some studies reported phytoremediation experiments with *Armeria maritima* on heavily contaminated areas. Ciarkowska reported Zn and Pb decrease in grounds contaminated with metals after *Armeria maritima* application

and suggested that this plant could be applied in phytostabilization (Ciarkowska et al. 2008). While Purmale et al. (2022) confirmed high tolerance to heavy metals (Pb, Zn, Cu, Cd) of soil-grown *Armeria maritima* in laboratory experiments. Despite the fact that *Armeria maritima* confirmed tolerance to high levels of heavy metals, it is not commonly used in large-scale phytoremediation processes (Reeves et al. 2006).

Phytoextraction is an eco-friendly and low-cost method but it has low efficiency because of slow plant growth and low mobility and bioavailability of metals in soil (Khan et al. 2000; Liu et al. 2020). There is a growing interest in improving new phytoextraction efficiency approaches. Recently, a promising technology enhancing phytoextraction has been approved based on plant-growth promoting bacteria (PGPB) use to enhance plant biomass production and tolerance to heavy metals. Among those PGPB there are rhizosphere microorganisms inhabiting plant roots (PGPR) and endophytes that inhabit internal plant tissues without causing them any harm (PGPE). PGPB protect plants and promote their growth mainly by producing antibiotics, phytohormones, by inducing plant ISR system, by dissolving some mineral nutrients like phosphorus or potassium and by iron chelating. PGPB can also stimulate heavy metal uptake and resistance by different mechanisms like for instance metal sorption, enzymatic reduction, oxidation or extracellular precipitation via active efflux pumping (Alves et al. 2022; Bargaz et al. 2018; Glich 2012; Klopper 1980; Kong et al. 2017).The most commonly used PGPB species are *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas* or *Rhizobium* (Alves et al. 2022).

PGPB have been used as biofertilizers in agriculture for a few decades. This is a very promising green technology that can replace or minimize chemical fertilizer use in agriculture. Nevertheless, recently many authors have pointed out that PGPB used as bioinoculants may enhance antibiotic resistance genes (ARGs) spread in soil because those microorganisms harbor ARGs very often (Chen et al. 2019; Mahdi et al. 2022; Zhang et al. 2020). ARGs can be located on mobile genetic elements (MGE) and they can be easily transferred among indigenous bacteria by horizontal gene transfer (HGT) (Arber 2014; Forsberg et al. 2012). Moreover, these promoting bacteria can harbour virulence factors causing potential risk for public health. There was proposed some putative regulatory framework that should be used for new bacterial-based biofertilizers. For instance, new biofertilizers should be well characterized (genome mining) regarding their AR profile, ARGs content and ARGs transfer potential. Moreover, multidrug resistant strains or human pathogens should be excluded. Finally, standard criteria, regulations and quality control procedures for biofertilizer candidates should be established, so as to guarantee environmental and public health protection (Mahdi et al. 2022).

Even though PGPB have been reported to carry antibiotic resistance genes, they are used in agriculture and they have been accepted as a new technique to assist phytoremediation. Experiments with PGPB used to enhance phytoremediation are mainly performed in laboratory conditions in pots or Petri dishes. For the last few years different strains of PGPB have been tested. For example, Wu et al. (2018) confirmed that an endophytic strain *Buttiauxella* sp. SaSR13 significantly enhanced cadmium accumulation in *Sedum alfredii* plant. Plant inoculation with this bacterium resulted in root elongation and stimulated secretion of organic acids and increased Cd uptake by *S. alfredii* as compared to the control plant after 7-day pot experiment. Endophytic assisted phytoremediation with *Sedum alfredii* was also investigated earlier by other authors (Zhang et al. 2013). They confirmed that *Burkholderia* sp., *Sphingomonas* sp., and *Variovorax* sp. strains significantly promoted Zn and Cd-extraction and have plant growth promoting properties. This experiment was conducted in pots for 60 days. Similar researches were performed by Wang et al. (2023) who revealed that inoculation of *Miscanthus floridulus* with an endophytic strain *Bacillus cereus* BL4 significantly strengthen Cd phytoremediation. Experiments were conducted in pots. Noteworthily, none of these PGPB were tested regarding antibiotic resistance profile.

In the presented study *Armeria maritima* spp. halleri endophytes were isolated and characterized regarding their heavy metal and antibiotic resistance properties. Bacteria inhabiting above ground parts of this plant have not been studied

so far. This study investigated new potential candidates as phytoextraction enhancing agents taking into account ARGs gene dissemination risk in accordance with the proposed regulatory frameworks for new bacterial biofertilizers mentioned in the Introduction section.

Materials and Methods

Study Site, sampling and soil physicochemical analysis

Studied area was located near ZGH “Bolesław” mining and metallurgical plant in Bukowno village, in the south of Poland (50°16'40.7"N 19°28'13.8"E). The sampling time was May 2015 at the flowering stage of the plants. Plant species selected for researches was *Armeria maritima* ssp. halleri. Collected plants were transferred in polyethylene bags and transported to the laboratory in an ice cooler at 4°C and tested within 2 days.

The total organic carbon, pH, calcium, magnesium, and heavy metal content (Cr, Cu, Cd, Ni, Pb, Zn, Hg) was determined: Hg content according to the procedure described in DIN ISO 16772, other heavy metals (ICP-OES/ICP-MS) – DIN EN ISO 11885/DIN EN ISO 17294-2, while pH was determined according to DIN EN ISO 10390 and TOC according to DIN EN ISO 15936.

Isolation and Purification of Heavy Metal Tolerant Bacteria

The heavy metal tolerant endophytic bacteria were isolated using the Luria Bertani agar (LB) medium supplemented with filter-sterilized soluble salts of lead $(CH_3COO)_2Pb$ (Pb^{2+}) or zinc $ZnSO_4$ (Zn^{2+}) in concentration of 20 mg/l. To isolate endophytic bacteria, the green parts of plants were separated and subjected to surface sterilization carried out in sterile conditions under a laminar chamber (Hung et al., 2004; Goryluk et al., 2009). Before starting this procedure, the ends of the stem sections were secured against the inflow of sterilization agents. The first stage of sterilization was rinsing the plant fragments in 70% ethanol for about 60 seconds, then transferring to 2% mercury chloride for 10 seconds and rinsing three times in distilled water. After surface sterilization, the plant material was homogenized and the obtained homogenates were 10-fold and 100-fold diluted and 0,1 ml aliquots were plated on culture media. All plates were incubated at 30° C for 24–48 hours. In order to determine the dry weight of the tested plants, each homogenate was poured onto a filter paper and weighed after complete drying. The obtained results made it possible to calculate the number of colony forming units per one gram of dry plant matter.

An individual bacterial colonies of different morphological characteristics were randomly selected and streaked on the LB agar medium supplemented with metal salts until pure cultures were obtained. A total of 100 bacterial isolates were selected for further studies and stored in 20% glycerol stock at -80 ºC.

Characterization of Heavy Metal Tolerant Bacteria

Identification

Morphological features of bacterial isolates (Gram staining) were carried out using light microscopy. Then, biochemical analyses were performed, which involved the activity of oxydase and catalase, gelatin hydrolysis, citrate utilization, glucose fermentation and production of urease and fluoresceine. Tests were prepared according to Bergey’s Manual of Systematic Bacteriology and identified to genus level (Claus and Berkeley 1986).

Five out of 100 Gram-negative bacterial isolates with different morphology were selected for further analysis. To evaluate species identification MALDI-TOF MS analysis was prepared. The standard Bruker interpretative criteria were applied. Score > 2.300 was used for certain species identification.

Heavy metals MIC assay

The Minimum Inhibitory Concentration (MIC) values were determined by the plate dilution method as adopted by Malik and Jaiswal (2000) with modifications. LB medium supplemented with filter-sterilized soluble salts of $(CH_3COO)_2Pb$ (Pb^{2+}) and $ZnSO_4$ (Zn^{2+}) was used. The starting concentration for each metal was 10 mM. Bacteria with density 10^6 CFU/ml were used for the inoculation. The MIC was taken as the lowest metal concentration that prevented the growth of the bacteria (Haroum et al. 2017). In this experiment *E. coli* 1655 strain was used as control (Spain and Alm 2003).

The antibiotic susceptibility test

The antibiotic susceptibility test was determined by disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing EUCAST version 11.0, valid from 2023-01-01. All 13 recommended for *Pseudomonas* spp. antibiotics were tested. Moreover, three antibiotics not included in EUCAST breakpoints were tested, such as fosfomycin (50 µg), streptomycine (25 µg) and erytromycine (15 µg). Zone diameter of bacterial growth inhibition around each of the antibiotic discs was interpreted according to the EUCAST criteria for *Pseudomonas* spp. If the bacterial genera or some antibiotic was not included in the standard, than lack of the inhibition zone was interpreted as no susceptibility to the given antibiotic.

Identification of the molecular resistance mechanisms

Genomic DNA of selected bacterial isolates was extracted according to Kpoda et al. (2018). DNA was stored at -20°C for subsequent use. Identification of efflux pumps coding genes was performed using PCR amplification method while isolation of *bla* genes was performed using Multiplex PCR.

PCR reaction targeting efflux pump genes *mexA* and *mex B* of the Mex AB-OprM pump was done as described elsewhere (Ugwuanyi et al. 2021). *MexD*, *mexF* and *mexY* genes of the MexCD-OprJ, MexEF-OprN, MexXY-OprM efflux pumps were amplified according to Poonsuk et al. (2014). Moreover, *czcA* and *czcR* genes encoding components of the CzcCBA efflux pump were amplified using primers proposed by Perron et al. (2004). To identify a type of ß-lactamase coding genes Multiplex PCR reactions were prepared (Colom et al. 2003; Dallene et al. 2010; Piotrowska et al. 2019). Four Multiplex PCR assays were performed for the detection of *bla* genes, such as bla_{TEM}, bla_{SHV} and bla_{OXA} genes (Multiplex I); bla_{CTXM} genes (Multiplex II); bla_{VER}, bla_{PES} and bla_{GES} genes (Multiplex III) and bla_{KPC}, bla_{IMP} and bla_{VIM} genes (MultiplexIV).

All the PCR amplicons were gel-purified (Gel-Out kit, AA Biotechnology) and submitted for sequencing using an ABI 3730 Genetic Analyzer (Applied Biosystems). Obtained gene fragments were searched against the National Center of Biotechnology Information (NCBI) using a local BLASTX program. A gene was designated as resistant gene if it had had at least 98% identity with other resistant genes in the database.

Whole Genome Sequencing and Bioinformatic Analysis

Genomic DNA from five selected bacteria was extracted using Genomic Mini® kit (A&A Biotechnology) as described by the manufacturer. DNA concentration and quality were checked with the Qubit^{TM}fluorometer (Invitrogen) and bacterial genomes were sequenced. The whole genome sequencing WGS was performed on MiSeq platform (Illumina) and the Unicycler version 0.4.8 assembly method was used. This Whole Genome Shotgun BioProject was deposited at DDBJ/ENA/GenBank under the accession number PRJNA886618.

TYGS Analysis

Strain genome sequences were uploaded to the Type Strain Genome Served (TYGS), that is a bioinformatic platform for digital, highly reliable estimator for relatedness of genomes based on DNA-DNA hybridization (DDH) (available at the website: http://tygs.dsmz.de) (Meier-Kolthoff et al. 2013).

RAST Analysis

Genomes were annotated in the Rapid Annotation using Subsystem Technology (RAST) server (available at the website: http://rast.nmpdr.org) (Aziz et al. 2008; Brettin et al. 2015; Overbeek et al. 2014). Annotation process enables the prediction of protein-coding genes, like ARG and HMRG, as well as other important elements, like direct and inverted repeats, insertion sequences, transposons and plasmids. To predict ARGs the Resistance Gene Identifier (RGI) application available at the Comprehensive Antibiotic Resistance Database (CARD) was also used (available at the website: http://card.mcmaster.ca/analyzer/rgi).

BLASTX Analysis

Obtained gene fragments were searched against the National Center of Biotechnology Information (NCBI) using a local BLASTX program. A gene was designated as an ARG or HMRG if it had at least 98% identity with its best hit in the database.

Results

Physio-chemical properties of soil

A total Pb concentration of 1100 mg of Pb in kg of soil^{-1} and a total Zn concentration of 3620 mg of Zn in kg of soil^{-1} was reported in studied site (Table 1). The given values allowed to classify the tested soil into the group of highly contaminated soils (Trafas et al. 2006). ZGH “Bolesław” S.A. is a Polish company existing from 1955 in Bukowno village, near Olkusz. Today, it is a modern mining and metallurgical complex, the main producer of zinc in Poland and the supplier of zinc to the neighboring countries, mainly to the Czech Republic, Slovakia, Austria and Hungary. In this plant zinc and lead ores are extracted and processed and electrolytic zinc, zinc alloys, sulfuric acid and zinc and lead concentrates are produced.

Table 1
Soil properties and metal concentrations

Soil properties		Total concentration of metals (mg/kg)								
pH	**TOC**	**Ca**	**Cd**	**Cr**	**Cu**	**Hg**	**Mg**	**Ni**	**Pb**	**Zn**
7.0	28 300	4530	35,00	8,40	18,50	0,15	2670	8,25	1100	3620

Biochemical Characterization of Heavy Metal Tolerant Bacteria

The mean total count of heavy metal tolerant bacteria isolated from A. maritima endosphere varied from 5.73 to 5.46 log CFU/g of plant material on lead and zinc supplemented medium, respectively. 100 selected for further researches isolates were Gram-negative, catalase positive and glucose fermentation negative. There were differences in urease and fluoresceine production test between isolates (Table 2). According to Bergey's Manual of Determinative Bacteriology isolates were identified as *Pseudomonas* spp. MALDI-TOF-MS analysis unable to species identification of isolates (Supp.Tab.S1).

Table 2
Biochemical characteristics of endophytic heavy metal tolerant bacteria

Isolate	Biochemical characteristics							
	Gram-staining	Oxidase	Catalase	Glucose fermentation	Gelatin liquefaciens	Citrate utilization	Urease	Fluoresceine production
AM4	negative	+	+	-	+	+	+	-
AM8	negative	+	+	-	+	+	+	-
AM14	negative	+	+	-	+	+	+	+
Z13	negative	+	+	-	+	+	+	-
Z18	negative	+	+	-	+	+	-	+

The antibiotic susceptibility test performed according to the EUCAST revealed different resistance profiles among selected isolates (Table 3, Supp. Tab.S2). All of the isolates were resistant to aztreonam (ATM), meropenem (MEM) and amoxicillin/clavulanic acid (AMC) while 4 out of 5 isolates were resistant to ceftazidime (CAZ), cefepime (FEP) and streptomycin (S). Only 2 isolates showed resistance to imipenem (IPM). Regarding heavy metal resistance a maximum MIC of 60 mM for Pb (II) was shown for 3 isolates (AM4, AM8, AM14) while a maximum MIC of 220 mM for Zn (II) was shown for 1 isolate, AM14 (Table 3). The lowest MIC (30 mM) was observed for isolate Z18 for both heavy metals tested.

Table 3
Antibiotic resistance profile and heavy metal minimum inhibitory concentration of endophytic bacteria isolated from Armeria maritima

Isolate	Antibiotic resistance profile							MIC (mM of metal ions)	
	AMC	ATM	CAZ	FEP	IPM	MEM	S	Pb (II)	Zn (II)
AM4	R	R	R	R	R	R	R	60	160
AM8	R	R	R	R	S	R	R	60	160
AM14	R	R	R	R	R	R	S	60	220
Z13	R	R	S	S	S	R	R	30	40
Z18	R	R	R	R	S	R	R	30	30
E.coli 1655								5	1

Resistance Determinants Detection

Multidrug efflux pumps encoding genes common in different *Pseudomonas* strains such as the Resistance Nodulation Cell Division family pumps genes (RND), were screened in tested bacteria. Nevertheless none of the Mex-type pumps genes were detected and only one of two tested CzcCBA system genes were detected by PCR in all tested strains. The PCR amplicons of the *czcR* gene were shorter (315 bp) than expected *czcR* gene (880 bp) and sequence analysis did not confirm membership to known resistance gene.

Regarding antibiotic resistance genes there were no results of Multiplex PCR amplifying *bla* genes.

Genome Characterization of Endophytic Pseudomonas **sp. Strains**

Multidrug resistant endophytic *Pseudomonas* sp. strains were sequenced on Illumina platform (AM4, AM8, AM14, Z13, Z18). The draft whole genome sequence length varied from 6.1 Mb to 7.4 Mb (Table 4). An average G + C content ranged from 60–61%. Annotation performed using RAST server predicted from 5,700 to 7,059 coding sequences. The analysis revealed from 374 subsystems with 65 RNAs genes to 395 subsystems with 63 RNAs genes. Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the following accessions: SAMN31135831, SAMN31136163, SAMN31136268, SAMN31137609, SAMN31137624 (Table 4).

Table 4
General features for *Pseudomonas* sp. strain draft genomes

Isolate	GenBank accession no.	Genome size (bp)	No. of contigs	No. of coding sequences	G + C content (%)	No. of subsystems	No. of RNAs genes
AM4	SAMN31135831	6,189,201	119	5,700	60.0	374	65
AM8	SAMN31136163	7,381,324	64	6,953	60.4	391	65
AM14	SAMN31136268	6,182,403	54	5,713	60.3	375	67
Z13	SAMN31137609	7,401,235	160	7,059	60.1	395	63
Z18	SAMN31137624	7,381,527	121	6,785	61.0	394	62

A whole-genome based taxonomic analysis based on DNA-DNA hybridization (in TYGS) demonstrated that isolate AM8 belongs to *Pseudomonas marginalis* species (dDDH above 80%) while the rest of the isolates cannot be identified as species (Supp. Tab.S3). Species with the highest homology to tested bacteria were, as follows, *P. paracarnis* (strain AM4, dDDH = 78,6%); *P. koreensis* (AM14, dDDH = 78,7%) and *P. yamanorum* (Z13, dDDH = 72,2%; Z18, dDDH = 70,7%).

Prediction of Heavy Metal Resistance Genes

All tested genomes contained homologues (100 – 98% homology) for the *copC*, *copD*, *copG*, *cueO*, *cusR* and *cusS* genes, which are essential for copper resistance (Table 5). *CopB* gene was detected in 3 genomes. Chromium resistance gene *chrA* homologues were detected in three genomes (AM4, AM8, AM14) while homologue of gene encoding cation diffusion facilitator transporter CzcD conferring resistance to cobalt, zinc and cadmium were found

in four tested *Pseudomonas* genomes (AM4, AM14, Z13, Z18). Moreover, gene *czcB* encoding one of the CzcCBA efflux transporter system and genes *czcR* and *czcS* encoding two-component regulatory system CzcRS were detected in three (AM8, Z13, Z18) and five tested genomes (AM4, AM8, AM14, Z13, Z18), respectively.

Table 5
Putative heavy metal resistance genes detected in tested *Pseudomonas* sp. genomes

Isolate	Heavy metal resistance gene											
	chrA	*copB*	*copC*	*copD*	*copG*	*cueO*	*cusR*	*cusS*	*czcB*	*czcD*	*czcR*	*czcS*
AM4	+	+	+	+	+	+	+	+	-	+	-	-
AM8	+	+	+	+	+	+	+	+	+	-	+	+
AM14	+	+	+	+	+	+	+	+	-	+	-	-
Z13	-	-	+	+	+	+	+	+	+	+	+	+
Z18	-	-	+	+	+	+	+	+	+	+	+	+

Genes encoding components of efflux pumps, extracellular sequestration proteins, P-type ATPases or metal detoxification proteins were detected in tested genomes (Table 6).

Table 6
Putative heavy metal resistance genes detected in tested *Pseudomonas* sp. genomes and their products

Detected gene	Gene product	Function	Resistance mechanisms	Heavy metal removed
chrA	Membrane protein	Metal efflux transporter	Chemiosmotic ChrA pump	Cr(VI)
copB	Membrane protein	Cation transport out of the cell	P-type ATPase	Cu(I)
copC	Periplasmic protein	Bind or transport Cu ions	P-type ATPases	Cu(I)
copD	Periplasmic protein	Bind or transport Cu ions	P-type ATPases	Cu(I)
copG	Periplasmic protein	Bind or transport Cu ions	P-type ATPases	Cu(I)
cueO	Periplasmic protein	Multicopper oxidase	Cue system	Cu(I)
cusR	Cytoplasmic protein	Regulator of the cusCFBA operon	CusCFBA efflux system	Cu(I)
cusS	Membrane protein	Regulator of the cusCFBA operon	CusCFBA efflux system	Cu(I)/Ag(I)
czcB	Membrane fusion protein	Divalent ion transport across the inner and outer membranes	CzcCBA efflux system RND-type pump	Co(II)/Zn(II)/ Cd(II)
czcD	Cytoplasmic membrane protein	Cation diffusion facilitator transporter	CDF protein family transporter	Co(II)/Zn(II)/ Cd(II)
czcR	Transcription regulator protein	Regulator of the czcCBA operon	CzcCBA efflux system	Co(II)/Zn(II)/ Cd(II)
czcS	Transcription regulator protein	Regulator of the czcCBA operon	CzcCBA efflux system	Co(II)/Zn(II)/ Cd(II)

Prediction of Antibiotic Resistance Genes

Genes conferring antibiotic resistance were detected in all tested *Pseudomonas* genomes (Table 7). *Bla*, *fosA* and *satA* genes responsible for antibiotic inactivation were detected as follows: bla genes in all tested genomes, satA gene in four genomes (AM4, AM8, Z13, Z18) while fosA gene in three genomes (AM4, AM8, AM14). Moreover, two genes responsible for antibiotic target alteration were present in all tested genomes, such as *gyrA and gyrB* genes with point mutation. Finally, genes coding different efflux pump components were identified in tested *Pseudomonas* genomes, such as *adeF*, *lysR*, *macA*, *norM*, *soxR* and *tolC* (Table 7).

Table 7
Putative antibiotic resistance genes detected in tested *Pseudomonas* sp. genomes

Isolate	Antibiotic resistance gene										
	adeF	*bla*	*fosA*	*gyrA*	*gyrB*	*satA*	*macA*	*norM*	*soxR*	*tolC*	*lysR*
AM4	+	+	+	+	+	+	+	+	+	+	-
AM8	+	+	+	+	+	+	+	+	+	-	-
AM14	+	+	+	+	+	-	+	+	+	-	+
Z13	+	+	-	+	+	+	+	+	+	-	-
Z18	+	+	-	+	+	+	+	+	+	+	-

Genes coding components of three antibiotic resistance mechanism types, such as antibiotic inactivation, antibiotic target alteration and antibiotic efflux pumps were identified in tested genomes (Table 8).

Table 8
Putative antibiotic resistance genes detected in tested *Pseudomonas* sp. genomes and their products

Detected gene	Gene product	Function	Resistance mechanisms	Antibiotics removed
bla	Metallo-ß-lactamases	Hydrolysis of the ß-lactam ring in antibiotic molecule	Antibiotic inactivation	β-lactams
fosA	FosA protein that is glutathione transferase	Addition of glutathione to antibiotic	Antibiotic inactivation	Fosfomycins
gyrA	Subunit A of the DNA gyrase	Point mutation in *gyrA*	Antibiotic target alteration	Fluoroquinolones
gyrB	Subunit B of the DNA gyrase	Point mutation in *gyrB*	Antibiotic target alteration	Fluoroquinolones
satA	N-acetyltransferase	Acetylation of streptothricin antibiotic	Antibiotic inactivation	Aminoglycosides
adeF	Membrane fusion protein AdeF	Component of the AdeFGH efflux pump	RND family efflux pump	Tetracyclines, fluoroquinolones
lysR	Transcription regulator LysR	Positive regulation of efflux pump gene expression	RND family efflux pump	Tetracyclines, fluoroquinolones
macA	Periplasmic membrane fusion protein MacA	Periplasmic fusion protein	ABC family efflux pump	Macrolides
norM	Transmembrane protein NorM	H^+ driven antiporter	MATE family efflux pump	Fluoroquinolones, gentamycin, trimetophrim, ciprofloxacin, norfloxacin, ethidium, kanamycin, streptomycin
soxR	Redox-sensitive transcriptional activator SoxR	Positive regulation of efflux pump gene expression	RND family MFS family ABC family efflux pumps	Tetracyclines, rifamycin, fluoroquinolones, cephalosporins, phenicols, penam, glycylcycline
tolC	Type I secretion outer membrane protein TolC	Component of efflux pumps	RND family MFS family ABC family efflux pumps	Tetracyclines, rifamycin, fluoroquinolones, cephalosporins, phenicols, penam, glycylcycline

Discussion

Antibiotic resistance genes present in endophytic bacteria are hardly tested. Some data are available for PGPB isolated from agricultural soil or crop plants. Nevertheless, ARGs detected in endophytes inhabiting green parts of metallophytes has not been described yet. Since the beginning of XXI century endophytes inhabiting metallophytic plants have gained attention (Idris et al. 2004). Up to the end of 2022 about 25 metallophytic plants were tested regarding endophytes (Alves et al. 2022; Goryluk-Salmonowicz et al. 2019). Endophytic microorganisms have plant growth promoting properties and they enhance heavy metal uptake by host plant. That is why they started to be considered as phytoremediation enhancing agents. In this study, for the first time endophytic bacteria have been isolated from *Armeria maritima* subsp. halleri growing on heavy metal contaminated soil. Tested metallophyte was grown on soil with high Pb and Zn concentrations, near to the ZGH "Bolesław" mining and metallurgical plant in Bukowno village, in the south of Poland (Table 1). Endophytes isolated from tested plants were resistant to lead and zinc and they were identified as *Pseudomonas* genus (Table 2). Two strains showed high MIC levels against Pb and Zn up to 60mM and 160mM, respectively (AM4 and AM8) while *Pseudomonas* AM14 strain was able to grow in extremely high Zn concentration in the medium, such as 200mM (Table 3). Several earlier studies also reported *Pseudomonas* sp. endophytes isolated from Pb and Zn accumulating plants. Sheng et al. (2008) isolated *Pseudomonas fluorescens* endophytes from *Brassica napus* and bacteria were resistant to lead and zinc, while Yongpisanphop et al. (2020) isolated lead resistant *Pseudomonas* sp. endophytes from *Pityrogramma calomelanos* metallophyte. *Pseudomonas* strains resistant to Pb or Zn were also isolated from soils. In 2021 Rehman et al. isolated zinc resistant *Pseudomonas oleovorans* ZSB13 strain and MIC for this strain was of 35mM while Jebara et al. (2015) isolated *Pseudomonas* sp. strains that tolerated up to 3.24 mM Pb. Antibiotic resistance genes were not tested in those researches.

Our findings (phenotypic antibiotic susceptibility test) revealed that tested bacteria were resistant to antibiotics belonging to two classes, such as aminoglycosides and β-lactam antibiotics (Table 3). All tested *Pseudomonas* strains were resistant to aztreonam, meropenem and amoxicilic clavunolic acid while only two strains were resistant to imipenem (*Pseudomonas* AM4 and AM14). Only one strain *Pseudomonas* Z13 was sensitive to cefalosporins, such as cefepime and ceftazidime and one strain *Pseudomonas* AM14 was sensitive to aminoglycoside antibiotic such as streptomycin.

To find resistance genes determinants in our strains the most common resistance mechanisms observed in *Pseudomonas* spp. were considered, such as RND efflux pump Mex-type systems and CzcCBA system (Lister et al. 2009; Perron et al. 2004; Piddock 2006; Poonsuk et al. 2014; Ugwuanyi et al. 2021). RND-type pumps act on a range of antibiotics and function as tripartite systems consisting of an outer membrane protein, periplasmic membrane fusion protein and a cytoplasmic membrane-associated RND transporter (Lorusso et al. 2022; Poonsuk et al. 2014; Ugwuanyi et al. 2021). Genes encoding efflux pump components are chromosomally located. In our strains neither membrane fusion protein coding gene *mexA* of the MexAB-OprM pump nor antiporter coding gene *mexB* was detected. Genes encoding other Mex-like pumps were also absent, such as *mexF*, *mexY* and *mexD*. Regarding CzcCBA system detected *czcR* gene didn't show homology to the known *czcR* genes and the PCR amplicons were shorter than expected. Tested strains were resistant to different ß-lactam antibiotics such as aztreonma, meropenem, imipenem, cefepime and ceftazidime. Therefore, searching for different variants of *bla* genes was performed. Nevertheless, there was no PCR product in tested bacterial samples.

Taking into account failure resistance genes identification by PCR we decided to sequence bacterial genomes (Table 4). Bioinformatics analysis of genomes and comparison with some known *Pseudomonas* strains enable to classify one strain as *Pseudomonas marginalis* AM8 and the rest bacteria were identified with high probability as *P. paracarnis* AM4 strain, *P. koreensis* AM14 strain and *P. yamanorum* (Z13 and Z18 strains) (Sup. Tab.S3).

A thorough search for genes coding for resistance to heavy metals confirmed that tested strains contain genes conferring resistance to copper, chromium, cobalt, zinc and cadmium (Table 5). In phenotypic test it was observed that tested endophytes were resistant to zinc and lead (Table 3). Genome analysis confirmed this finding and genes implicated in zinc resistance were identified (Table 5). Gene *czcD* encodes cation diffusion facilitator transporter CzcD, gene *czcB* encodes one of the CzcCBA efflux transporter system while genes *czcR* and *czcS* encode two-component regulatory system CzcRS. Those genes are a part of the Czc system that is composed of CzcCBA transporter across the inner and outer membrane regulated by 2-component system CzcRS and CzcD cation diffusion facilitator protein (CDF). The CzcCBA system functions as a cation-proton antiporter and it transfers Cd^{2+}, Co^{2+} and Zn^{2+} ions outside the bacterial cell (Liu et al., 2005; Rensing et al., 1997; Wang et al., 2017). Genomes of *Pseudomonas yamanorum* Z13 and Z18 strains contained *czcB*, *czcD*, *czcR* and *czcS* genes. In the *P. marginalis* AM8 strain there were 3 genes detected, such as *czcB*, *czcR* and *czcS* while two other genomes of *Pseudomonas* (AM4 and AM14) contained only 1 gene, such as *czcD*. To compare our results with results obtained by other authors we decided to analyse published genomes of other environmental *Pseudomonas* strains isolated from plant roots or green parts of plants (Table 9). The results confirmed that the CzcCBA system is not always present in environmental bacteria. For instance, in the genome of *Pseudomonas fluorescens* Pf-5 isolated from cotton rhizosphere (Howell et al. 1979; Paulsen et al. 2005) there were no genes encoding CzcCBA system. While the analysis of three other genomes of endophytic *Pseudomonas* sp. strains confirmed presence of the CzcCBA system genes in *P. poae* A2-S9 strain isolated from *Panicum virgatum* plant, *Pseudomonas* sp.382 strain isolated from *Paullinia cupana* seeds (de Siqueira et al 2018; Liotti et al. 2018; Xia et al. 2013; Xia et al. 2019)d *putida* GM4FR endophyte isolated from green parts of *Festuca rubra* L. (Wemheuer et al. 2016, 2017).

Table 9
A list of plant growth promoting environmental *Pseudomonas* sp. strains with sequenced genomes (whole/draft genomes)

Strain	Host	Isolation source	Genome/ No. of contigs	References
P. atacamensis M7D1	Desert bloom plants	Rhizosphere	6.2 Mb/ 12	Poblete-Morales et al., 2019
P. chlororaphis GP72	*Piper* L. (green pepper)	Rhizosphere	6.6 Mb/ 347	Shen et al., 2012; Liu et al., 2006
P. fluorescens Pf-5	*Gossypium hirsutum* (cotton)	Rhizosphere	7.1 Mb/ 1	Howell et al., 1979; Paulsen et al., 2005
P. fluorescens UM270	*Medicago truncatula*	Rhizosphere	6.0 Mb/ 524	Salmeron et al., 2016; Hernández-León et al., 2015
P. poae A2-S9	*Panicum virgatum*	Plant endosphere	6.68 Mb/ 3	Xia et al., 2013; Xia et al., 2019;
P. putida GM4FR	*Festuca rubra* L.	Plant endoshere (aerial parts)	7.1 Mb/ 79	Wemheuer et al., 2017; Wemheuer et al., 2016
P. viridiflava CDRTc14	*Lepidium draba*	Plant endoshere (roots)	6.7 Mb/ 38	Samad et al., 2016
Pseudomonas sp. RGM-2987	*Stevia philippiana*	Rhizosphere	6.1 Mb/ 223	Guerra et al., 2023
Pseudomonas sp. SH-C52	Sugar beet plant	Rhizosphere	6.3 Mb/ 384	Mendes et al., 2011; Van Der Voort et al., 2015
Pseudomonas sp.382	*Paullinia cupana*	Plant endosphere (seeds)	6.38 Mbp/ 115	de Siqueira et al, 2018; Liotti et al., 2018

In tested bacterial genomes chromium and copper resistance genes were detected (Table 5). Three *Pseudomonas* strains (AM4, AM8, AM14) contained **c**hromium resistance gene *chrA* which encodes membrane transporter protein responsible for efflux of chromium out of the cell cytoplasm (Branco et al. 2008). ChrA is a membrane potential dependent transporter and it was detected in a variety of bacterial genera, like *Arthrobacter* spp., *Bacillus* spp., *Lysinibacillus* spp. or *Pseudomonas* spp. (He et al. 2010, 2011; Henne et al. 2009; Mondaca et al. 1998). *ChrA* gene was also present in all four mentioned above environmental *Pseudomonas* genomes described by other authors and in other strains, like *P. chlororaphis* GP72 isolated from green pepper rhizosphere, *P. viridiflava* CDRTc14 isolated from roots of *Lepidium draba* and *P. fluorescens* UM270 isolated from roots of *Medicago truncatula* (Table 9) (Hernández-León et al. 2015; Samad et al. 2016; Liu et al. 2006; Salmeron et al. 2016; Shen et al. 2012).

In our study, all tested genomes contained *copC*, *copD* and *copG* genes encoding periplasmic proteins that bind and/or transport Cu ions and three genomes such as AM4, AM8 and AM14 contained *copB* gene coding outer membrane protein. CopB protein is responsible for Cu transport out of the cell (Outten et al. 2001; Marrero et al. 2012; Zhang et al. 2006) Interestingly, CopG is a commonly detected protein present in Gram-negative bacteria resistant to copper but it is an uncharacterized protein. In 2020 Hausrath proposed that CopG is an oxidoreductase and it converts Cu(I) and Cu(II) to facilitate export of copper by the Cus RND transporter efflux system (Hausrath et al. 2020). *Cop B, copC*, *copD* and *copG* and *copG* genes were also present in all mentioned environmental *Pseudomonas* sp. genomes (Table 9).

All tested in this study bacterial genomes contained *cueO* gene encoding multicopper oxidase and *cusR* and *cusS* genes encoding regulatory proteins of the CusCFBA system involved in periplasmic copper detoxification. CusS is a sensor histidine kinase while CusR is a regulatory protein. C*opG*, *cueO, cusR* and *cusS* genes were also detected in other environmental Pseudomonas strains (Table 9).

Heavy metal resistance properties of environmental bacteria give them the applicable potential. They can be used in different biotechnologies as environmentally safe alternatives. Nevertheless, metal resistant bacteria are very often resistant to other toxic compounds like antibiotics (Baker-Austin et al. 2006; Goryluk-Salmonowicz and Popowska 2019; Seiler and Berendonk 2012). Genes conferring resistance can be located on mobile genetic elements and easily transfer among other bacteria (Baker-Austin et al. 2006; Zhang et al. 2020). Taking into account that tested in this study bacteria could be used in biotechnology we decided to study antibiotic resistant profile of selected *A. maritima* inhabiting endophytes (Table 3) and identify resistance genes (Table 8).

Three genes responsible of antibiotic inactivation were found in tested genomes, such as *bla*, *fosA* and *satA* gene. Resistance to ß-lactam antibiotics is largely mediated by enzymes called ß-lactamases encoded by *bla* genes. Those enzymes are divided in four classes (A, B, C, D) and form two groups, such as metallo-ß-lactamases and serine ß-lactamases. Metallo-ß-lactamases belong to Class B. Class B is divided in three subclasses, chromosomally encoded genes belong to B2 or B3, while MGE-encoded genes belong to the B1 subclass (Boyd et al., 2020). In our studies all tested genomes contained *bla* genes (Table 7). This result was consistent with antibiotic resistance profile as tested bacteria were resistant to ß-lactam antibiotics (Table 3). Sequences comparison of identified genes with some other *bla* genes in the NCBI database revealed that they belong to metallo-ß-lactamases. There is a high probability that *bla* genes belong to the chromosomally encoded subclass B2 or B3 ß-lactamases.

The second gene implicated in antibiotic inactivation mechanism detected in tested genomes was *fosA*. *FosA* gene encodes FosA protein and it is the most common fosfomycin resistant mechanisms in Gram-negative bacteria. FosA is a Mn^{2+}, K^{+} dependent metalloenzyme that catalyzes addition of glutathione to fosfomycin resulting in antibiotic inactivation. Gene *fosA* can be localized on plasmids or chromosomes of bacteria. Plasmid-borne *fosA* genes are mainly reported in fosfomycin resistant *Escherichia coli* strains (Castañeda-García et al. 2013; Ito et al. 2017). In tested genomes *fosA* gene was detected in *Pseudomonas* sp. AM4, AM8 and AM14 strains (Table 7). These results are in accordance with antibiotic resistance profile for these 3 strains because the inhibition zone around antibiotic disk was narrow (< 22 mm) comparing to other two strains (> 40mm) (Supp. Tab.I). Similar results were described by Ito et al. (2017) who prepared susceptibility test of *E. coli* TOP10 transformants harboring chromosomal *fosA* gene from *P. aeruginosa* PAO1 strain and get 22mm diameter of inhibition zone.

Finally, *satA* gene was detected in four out of five tested genomes (all without *Pseudomonas* sp. AM14). *SatA* gene encodes GCN5-related N-acetyltransferase (GNAT) that is responsible for acetylation and inactivation of streptothricin antibiotic (Burckhardt et al., 2019). Streptothricin is a broad-spectrum aminoglycoside antibiotic produced by soil microorganisms. This antibiotic is rarely used clinically because of its high toxicity. *SatA* gene in environmental bacteria had been detected earlier by other authors. For instance, Burckhardt et al. (2017) detected *satA* gene in *Bacillus subtilis* 168.

Bla, *fosA* and *satA* genes with high homology to tested genes were also present in some other environmental *Pseudomonas* strains (Table 9). While *bla* gene was present in all mentioned above *Pseudomonas* strains, *fosA* and *satA* genes were present in some of them only. For instance, only *satA* gene was present in *P. fluorescens* Pf-5 strain while both genes were observed in *P. poae* A2-S9, *P. chlororaphis* GP72 or *Pseudomonas* sp. RGM-2987 isolated from *Stevia philippiana* roots (Guerra et al. 2023).

Two genes responsible for antibiotic target alteration were detected in all tested strains, such as *gyrA* and *gyrB*. Antibiotic target alteration mechanism consist of a single point mutation in *gyrA* and *gyrB* genes that leads to lowered affinity between antibiotic and its target. This is a very common fluoroquinolones resistance mechanism detected in Gram-negative bacteria. Mutations in quinolone-resistance determining regions, such as *gyrA* or *gyrB* in DNA gyrase are chromosomaly encoded. Researches indicated that high-level resistance to fluoroquinolones required mutations in at least two genes with quinolone-resistance determining regions (Weigel et al. 1998; Zhang et al. 2015). In our studies all tested genomes contained a single point mutations in *gyrA* and *gyrB* genes. Our findings revealed also (phenotypic antibiotic susceptibility test) that the inhibition zone around fluoroquinolones (CIP, LEV) is narrower for all strains (< 30mm) than the susceptibility zone defined by EUCAST (> 50mm) (Supp. Tab.S2). Single point mutations in *gyrA* and *gyrB* genes were also observed in other environmental *Pseudomonas* sp. strains (Table 9)..

Finally, six genes coding efflux pump components were identified in tested genomes, such as *adeF*, *lysR*, *macA*, *norM*, *soxR* and *tolC* (Table 7, 8).

AdeF protein is a component of the AdeFGH pump and it is a membrane fusion protein. AdeFGH belongs to the RND type efflux pump (Coyne et al. 2010). AdeFGH efflux pump confer resistance to tetracycline and fluoroquinolone antibiotics. LysR protein is a transcriptional regulator and it regulates expression of the AdeFGH efflux pump genes. High expression of this pump confer resistance to chloramphenicol, clindamycin, fluoroquinolones, and trimethoprim. *AdeF* gene and *lysR* genes were identified in all tested strains. AdeFGH pump is a commonly detected system in *Acinetobacter baumanii* clinical isolates but not in *Pseudomonas* strains (Coyne et al. 2011; He et al. 2015; Rumbo et al. 2013). The closest analog of the AdeFGH pump is a MexEF-OprN pump commonly detected in *Pseudomonas aeruginosa* clinical strains (Maseda et al. 2000; Uwate et al. 2013). MexEF-OprN pump was detected also in *P. fluorescens* Pf-5 strain isolated from rhizosphere. Nevertheless, the results of our studies excluded the presence of the MexEF-OorN pump because there was no PCR product with primers amplifying *mexF* gene. Further researches need to be done in order to explain this mechanism.

In tested genomes genes involved in different type efflux pump component production were also detected, such as *sox*R and *tolC*. *TolC* gene, which were detected in two strains such as *Pseudomonas* AM4 and *Pseudomonas* Z18 while *soxR* gene was present in all genomes. TolC protein is an outer membrane transporter and it is a component of different types of efflux pumps (Greene et al. 2018). The redox-sensitive protein SoxR is a global regulator of different efflux pumps gene expression. It belongs to the MerR-family transcriptional regulators and it is common among Gram-negative and Gram-positive bacteria. In enteric bacteria SoxR mediates resistance to oxidative stress caused by nitric oxide or superoxide and it induces the expression of the *soxS* gene. SoxS activates transcription of more than 100 genes encoding products that repair cellular damage. In nonenteric bacteria like *Pseudomonas* sp. SoxR directly activates transcription of several multidrug efflux pump genes conferring resistance to antibiotics (Park et al. 2006). One of the pump is MexGHI-OmpD efflux pump detected commonly in *Pseudomonas aeruginosa* clinical strains. MexGHI-OpmD confer resistance to acriflavine, norfloxacin, tetracycline and vanadium and belongs to the RND type efflux pump (Aendekerk et al. 2002; Sekiya et al. 2003). Further analyses need to be done to explain if tested bacteria express MexGHI-OmpD efflux mechanism.

Two more genes connected with efflux pump production were detected, such as *macA* and *norM*. MacA is a periplasmic membrane fusion protein that is a component of the MacAB-TolC tripartite efflux pump and it stimulates MacB ATPase. MacAB-TolC belongs to the ABC superfamily, ATP Binding Casette transporter efflux pump (Crow et al., 2017). It is involved in substrate transport through a TolC-like channel. MacAB-TolC pump confers resistance to macrolides. In our genomes *macA* gene was present in all strains and strains were resistant to erythromycin (Supp. Tab.S2). It is highly probable that tested strains contain MacAB-TolC efflux pump. MacAB-TolC system was also

detected in other environmental bacterial genomes, like for instance in *P. fluorescens* Pf-5 genome *P. chlororaphis* GP72 or *Pseudomonas* RGM-2987 (Table 9). It was also observed in *Pseudomonas* sp. SH-C52 strain isolated from sugar beet roots and *P. atacamensis* M7D1 isolated from roots of desert bloom plants from the Atacama region (Mendes et al. 2011; Poblete-Morales et al. 2019; Van Der Voort et al. 2015).

NorM is a multidrug transporter categorized in the MATE (Multidrug and toxic compound extrusion) family (Morita et al. 2000; Rouquette-Loughlin et al. 2003). NorM is a H^+ -driven H^+ /drug antiporter and it confers resistance to different antibiotics (Table 8). In our study, in all genomes we identified a gene that could encode a protein highly similar to NorM protein detected in other environmental *Pseudomonas* strains. That protein was also detected in *Pseudomonas* SH-C52, in *Pseudomonas* RGM-2987 or in *Pseudomonas putida* GM4FR (Table 9).

Conclusion

The present study demonstrated that *Armeria maritima* subsp. halleri is inhabited by resistant to heavy metals and antibiotic endophytes, identified as *Pseudomonas*. The bacteria contained heavy metal markers in their genomes conferring resistance to cadmium, cobalt, copper, chromium and zinc. Genes encoding components of efflux pumps, extracellular sequestration proteins, P-type ATPases or metal detoxification proteins were detected. Moreover, the bacteria were resistant to antibiotics. The resistance concerned such antibiotics as: aminoglycosides, β-lactams, fosfomycines, fluorochinolones, macrolides and glycopeptides. Genes encoding three antibiotic resistance mechanism types, such as antibiotic inactivation, antibiotic target alteration and antibiotic efflux pumps were identified.

Antibiotic resistance genes spread in the environment is a global problem and soil is considered to be the main reservoir of ARGs. Soil bacteria and plant associated bacteria are recipients of ARGs and form a part of the transmission routes for resistant genes. Endophytes inhabiting hyperaccumulators are beneficial for host plant but they can harbor antibiotic resistance genes (ARGs). In the available literature, definitely less attention has been given to the problem of antibiotic resistant plant growth promoting bacteria used in agriculture while resistance profiles of endophytes used in phytoremediation were not considered at all. Noteworthily, latest agricultural management system promotes the use of biological control agents and biofertilizers. The number of biopreparations available on the market is still growing but large-scale and long-term usage of biopreparations containing ARGs may lead to dissemination of antibiotic resistance.

Declarations

Acknowledgements

We are grateful to Professor Mieczysław Błaszczyk for his assistance in the fieldwork. This work was supported by the National Science Centre and the national Ministry of Education and Science.

Funding

This work was supported by the National Science Centre (NCN), Poland (UMO-2019/32/Z/NZ8/0011), international project in the frame of the Biodiversity and its influence on human, animal and plant health (BiodivERsA Call 2018): project “ANTIVERSA – Biodiversity as an ecological barrier for the spread of clinically relevant antibiotic resistance in the environment” to MP and partially in the frame of the ‘Excellence Initiative-Research University (2020-2026)’ Program at the University of Warsaw. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Competing Interests

The authors declare that they have no competing interests.

Author Contribution

A.G-S. – Conceptualization, Investigation, Formal Analysis, Writing, Funding acquisition, Original Draft, Visualization; AW.M. – Investigation; M.P. – Writing, Review & Editing, Supervision, Funding Acquisition, Project Administration.

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

The authors declare that they have no conflict of interest.

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