Screening the cultivable cave microbial mats for the production of antimicrobial compounds and antibiotic resistance

Jerneja Ambrožič Avguštin¹, Patricia Petrič¹, and Lejla Pašić^{1,2*}

¹Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

²School of Medicine, University Sarajevo School of Science and Technology, Hrasnička cesta 3a, 71000 Sarajevo, Bosnia and Herzegovina

Abstract: The current work extends the phenotypic characterization of a bacterial culture collection obtained from white, yellow, grey or pink microbial cave wall colonies that are common in the caves of Slovenian Karst. We have determined antibiotic resistance to 22 natural and synthetic antibiotics in 69 isolates from the microbial mats. Thirty-eight isolates (52%) were resistant to 1-5 antibiotics; another 27 isolates (37%) were resistant to 6-10 antibiotics; and 7 isolates (0.1%) were resistant to 11-17 antibiotics. We screened for production of antimicrobial compounds by growing cave isolates on five different media and overlaying individual cultures with ten Gram-positive and Gram negative multidrug resistant isolates. Out of 78 isolates tested, 15.3% showed antimicrobial activity against *Escherichia coli* DH5, 15.3% against extended-spectrum β -lactamase producing *Escherichia coli*, 3.8% against *Salmonella enterica* serovar Typhimurium TL747, 9% against *Klebsiella pneumoniae* ATCC BAA-1706, 9% against carbapenem-resistant *Klebsiella pneumoniae* ATCC BAA-1705, 7.7% against *Bacillus cereus*, 20.5% against *Bacillus subtilis*, 9% against *Listeria monocytogenes*, 19.2% against methicillin-resistant *Staphylococcus aureus*, and 20.5% against methicillin-resistant *Staphylococcus pseudointermedius*. Results showed the potential of cave microbes to suppress the growth of multi-resistant pathogens, and, a relatively high resistance to antibiotics.

Keywords: caves, microbial mats, microbiota, antimicrobial activity, antibiotic resistance

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INTRODUCTION

The discovery of antibiotics and their successful clinical use is considered the most significant development in the history of medicine. However, the acquisition and spread of antibiotic resistance genes among pathogenic bacteria has dramatically reduced the choice and effectiveness of many antibiotics available today for the treatment of infections in humans and animals.

The production of antibiotics and resistance determinants is ubiquitous in bacteria in which resistance gene products neutralize or detoxify the secondary metabolites produced by the same or neighboring cells. Resistance genes are often linked to and coregulated with the antimicrobial biosynthesis genes. Hence, the widespread occurrence of antibiotic resistance in environmental bacteria is an important indicator of antibiotic production, as seen in soil (D'Costa et al., 2006; Nodwell, 2007).

Environmental microbes remain an important source of novel bioactive compounds due to their ability to spread and colonize other environments, organisms, and along the food chain and facilitate the resistance transfer to other pathogens (Martínez, 2008; Wright, 2010; Bondarczuk et al., 2016; Cychón et al., 2019). Thus, it is important to establish surveillance and monitoring of environmental resistances as recently proposed by WHO as part of a One-health approach (McEwen & Collignon, 2018).

Today, more than 80 percent of chemotherapeutic antibiotics originate from secondary metabolism from well-studied soil *Actinobacteria* (de Lima Procópio et al., 2012). Over the past decades, the discovery rate of natural antibiotics has been steadily declining, moving the search for novel bioactive molecules towards other environments, including those considered 'extreme' from the anthropocentric point of view.

In this respect we have decided to supplement the diversity data of microbial mats that colonize the

*lejla.pasic@ssst.edu.ba

walls of a karst cave (Herzog-Velikonja et al., 2014) and extend the phenotypic characterization of these bacterial isolates from the culture collection. Caves are considered extreme environments due to absence of light, lack of direct contact with the surface primary production, permanent climatic conditions, and scarce and often highly complex nutrients (Lee et al., 2012).

Previous culture-independent studies have established that cave microbial mat communities are dominated by yet-to-be-cultivated members of *Actinobacteria* and *Proteobacteria* (Pašić et al., 2009; Porca et al., 2012). Cultivation-dependent approach has resulted in a description of 80 unique isolates, most of which could not be affiliated with validly described species and hence likely represent novel taxa (Herzog-Velikonja et al., 2014).

In this study, we hypothesized that the cave conditions impact the growth of cave mat bacteria in the form of delimited mat communities. This would result in high local production of antimicrobials and corresponding high resistance to these compounds. This gives a great potential for further use of isolated strains as a source of novel bioactive compounds.

To establish the potential of cave microbes as an antimicrobial resistance source we evaluated the levels

of antibiotic resistance to 22 natural and synthetic antibiotics. To establish the potential of cave microbes as a source of novel antimicrobial compounds, we determined their bactericidal activity against ten selected Gram-positive and Gram-negative multidrug resistant clinical isolates.

MATERIALS AND METHODS

Origin of the isolate collection

Bacterial strains were isolated from yellow, grey, and pink microbial mats from the walls of Pajsarjeva jama (Pajsar Cave) (Fig. 1A) and taxonomically evaluated in a previous study (Herzog-Velikonja et al., 2014). Their taxonomic affiliation is presented in Figure 1B. Pajsarjeva jama is located 20 km southwest of Ljubljana, Slovenia (45°49'51"N, 14°16'15"E), is 555 m long, and contains a small stream that flows underground through the cave. Its air temperature is 12°C with minor oscillations year-round, and the humidity reaches 100%. The 80 unique isolates were obtained in pure cultures on five different growth media with different nutrient concentration as described previously (Herzog-Velikonja et al., 2014). The isolates are preserved in culture collection at Biotechnical Faculty, University of Ljubljana, Slovenia.

Fig. 1. A) Microbial mats in Pajsarjeva jama under stereomicroscope; photo: L. Pašić, B. Sket; B) Maximum likelihood tree depicting phylogenetic positioning of 80 Pajsarjeva jama isolates belonging to Actinobacteria (green), Firmicutes (grey), and Proteobacteria (red).

Screening the Pajsarjeva jama isolate collection for the presence of antibiotic resistance

Bacterial cultures that were grown for 48 h at 30°C (Laiz et al., 2003) in Mueller-Hinton liquid medium were diluted to match a 0.5 McFarland turbidity standard. To obtain uniform growth, we streaked 4 mm-thick Mueller-Hinton agar plates three times with the cotton swab, rotating the plate for 90° in between

streaks. The plates were air-dried under sterile conditions for 5 min and discs impregnated with antibiotics (Hardy Diagnostics, Oxoid™) were then placed on the surface. To comply with manufacturer's instructions, the plates were left at room temperature for 15 mins and were then incubated for 24 h at 37°C. We then determined the diameter of the zones of inhibition and classified cultures as resistant as

per manufacturer's instructions (Hardy Diagnostics, Oxoid™). We determined the levels of resistance to 22 natural and synthetic antibiotics, whose characteristics are presented in Table 1.

Screening the Pajsarjeva jama isolate collection for antimicrobial activity

Cultivation conditions

The Pajsarjeva jama bacterial isolates, preserved as glycerol stocks, were plated onto tryptic soy agar (Difco,

USA) in quintuplicates and incubated for 48 h at 30°C as suggested by Laiz et al. (2003). Plates containing individual isolates were subsequently replica plated onto five different growth media in triplicates and incubated for further 48 h at 30°C. The five media were chosen based on their different ability to elicit the production of antibiotics and were Mueller-Hinton agar (Difco, USA), M9 minimal medium with glucose agar, tryptic soy agar (Difco, USA), Luria-Bertani (LB) agar (Difco, USA), and Nutrient agar (Difco, USA).

Table 1. Characteristics of antibiotics used in this study.

Mode of action		Antibiotic	Action	Resistance*	Disk potency (µg)
Inhibitors of cell-wall synthesis					
β -lactams		Ampicillin	Natural	Variable	10
		Penicillin	Natural	Variable	10
Modified β-lactams	2 nd generation cephalosporine	Cefaclor	Synthetic	Variable	30
	3 rd generation cephalosporine	Cefotaxime	Synthetic	Moderate	30
		Ceftazidime	Synthetic	Moderate	30
	3 rd generation cephalosporine	Cefpodoxime	Synthetic	Moderate	10
	4 th generation cephalosporine	Cefepime	Synthetic	Rare	30
	carbapenem	Ertapenem	Synthetic	Moderate	10
	carbapenem	Imipenem	Synthetic	Moderate	10
Inhibitors of protein synthesis					
Macrolides		Azithromycin	Synthetic	Moderate	15
		Erythromycin	Natural	Moderate	15
		Tobramycin	Natural	Widespread	10
Aminoglycosides		Gentamicin	Natural	Widespread	10
		Chloramphenicol	Natural	Moderate	30
		Florfenicol	Synthetic	Moderate	30
Lincosamide		Clindamycin	Synthetic	Moderate	2
Inhibitors of DNA/RNA synthesis					
Quinolones		Ciprofloxacin	Synthetic	Rare	5
		Nalidixic acid	Synthetic	Rare	30
		Metronidazole	Synthetic	Rare	25
Inhibitor of DNA synthesis, breaks down DNA		Rifampin	Natural	Moderate	5
Inhibitor of RNA synthesis		Trimethoprim	Synthetic	Moderate	5
Inhibitor of folic acid synthesis		Nitrofurantoin	Synthetic	Rare	300

*The Center for Disease Dynamics Economics and Policy. ResistanceMap: Antibiotic resistance. 2019. <https://resistancemap.cddep.org/AntibioticResistance.php>. Date accessed: May 31, 2019.

The production of antimicrobial compounds by cave isolates was screened as follows. Antibiotic-resistant indicator strains were grown overnight at 37°C in 10 ml of liquid LB medium. We used ten indicator strains. Strains *Klebsiella pneumoniae* ATCC® BAA-1706™ and carbapenem-resistant *Klebsiella pneumoniae* ATCC® BAA-1705™ were obtained from the American Type Culture Collection (USA). Strains *Escherichia coli* DH5 (no antibiotic production), *Salmonella enterica* serovar Typhimurium TL747, *Bacillus cereus* and *Bacillus subtilis* were obtained from the Genetic Laboratory Microbe Culture Collection at Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia. Strains extended-spectrum β-lactamase producing *Escherichia coli* Ž39, *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus*

pseudointermedius were obtained from the Institute of Microbiology and Parasitology at Veterinary Faculty, University of Ljubljana, Slovenia.

Antimicrobial activity screens

The production of antimicrobial compounds from Pajsarjeva jama isolate collection was established using soft-agar overlay technique (Hockett & Baltrus, 2017). Briefly, the Pajsarjeva jama isolate cultures were grown on agar for 48 h at 30°C, upon which the colonies were lysed by exposure to chloroform vapors for 10 min. Residual chloroform was removed by airing the plates for 20 min, and the cells were overlaid with 3 ml of soft agar that contained 200 µl of overnight culture of respective indicator strain. The prepared plates were incubated at 37°C for 24 h upon which we recorded the occurrence and the diameter of growth inhibition zones.

Statistical analysis

In order to identify growth media with significant effects on antibiotic production, we collected the data on antibiotic production on different growth media, we then log(10) transformed the data, performed one-way ANOVA and calculated the difference between each pair of means using Fisher's least significant difference procedure as implemented in Excel software.

RESULTS

Antibiotic resistance screen of Pajsarjeva jama isolates

We conducted antibiotic resistance screens on 69 isolates (71% Gram-positive; 29% Gram-negative) from our culture collection that grew on Muller-Hinton agar. While resistance to most antibiotics differed with the organisms tested, all organisms were sensitive to rifampin.

Antibiotic resistance in Gram-positive isolates

Resistance profile for 48 Gram-positive isolates is shown in Figure 2. Resistance to the cell-wall inhibitors was lowest for carbapenem antibiotic imipenem (4.1%) and cefepime (8.2%), a 4th generation cephalosporine. Gram-positive isolates were also highly resistant to

modified beta-lactam antibiotics, where resistance levels ranged from 53.1% (cefaclor) to 65.3% (cefepodoxime).

Within the protein synthesis inhibitor group, isolates were least resistant to aminoglycoside gentamicin (2.0% resistant isolates) and macrolide azithromycin (4.1% resistant isolates). The highest levels of resistance were recorded for aminoglycosides florfenicol (59.2%) and clindamycin (65.3%). Isolates were also highly resistant to DNA synthesis inhibitors particularly quinolones, i.e. nalidixic acid (79.6%) and metronidazole (87.8%), while the resistance levels of inhibitor of RNA synthesis trimethoprim (44.9%) and folic acid inhibitor nitrofurantoin (40.1%) were in intermediate range.

With the exception of two *Streptomyces* isolates that were sensitive to all antibiotics, all Gram-positive isolates were classified as multi-resistant, that is resistant to at least two antibiotics belonging to different chemical classes (Godebo et al., 2013; Exner et al., 2017). Ten isolates were resistant to 1-5 antibiotics; another 19 isolates were resistant to 6-10 antibiotics; and 17 isolates were resistant to 11-17 antibiotics. Within the latter group, isolate *Oerskovia* sp. B22 was resistant to 17 antibiotics, and *Micrococcus* sp. B43 was resistant to 15 antibiotics (Table 2).

Fig. 2. Antibiotic resistance levels in Pajsarjeva jama isolates belonging to Gram-positive bacteria against various antibiotics.

Table 2. Range of antibiotic resistance to different antibiotics in Gram-positive isolates with respect to isolate genus.

Genus	Number of isolates	Number of resistances per isolate
<i>Aerococcus</i>	2	8, 14
<i>Agrococcus</i>	1	4
<i>Arthrobacter</i>	2	5, 11
<i>Bacillus</i>	1	7
<i>Kocuria</i>	2	6, 14
<i>Microbacterium</i>	2	11, 13
<i>Micrococcus</i>	9	5, 6, 6, 7, 8, 10, 13, 14, 15
<i>Oerskovia</i>	2	14, 17
<i>Paenibacillus</i>	2	7, 11
<i>Rhodococcus</i>	8	7, 7, 7, 7, 8, 11, 11, 13
<i>Streptomyces</i>	17	0, 0, 3, 3, 4, 4, 4, 4, 7, 9, 10, 10, 11, 11, 11, 11, 12

Antibiotic resistance in Gram-negative isolates

Resistance profile for 19 Gram-negative isolates is shown in Figure 3. Levels of resistance to inhibitors of cell-wall synthesis ranged from 5.3% for cefepime to 78.9% for cefaclor. Among inhibitors of protein synthesis, isolates were highly resistant to macrolide erythromycin (73.6%) and lincosamide clindamycin (78.9%), and most sensitive to gentamicin (21.1% resistant isolates). Among inhibitors of DNA/RNA synthesis, the isolates were most resistant to quinolone metronidazole (89.5% resistant isolates), and least resistant to ciprofloxacin (21.1% resistant isolates).

All Gram-negative isolates were resistant to at least four antibiotics from two different groups and were classified as multi-resistant. Two isolates were resistant to 4 and 5 antibiotics, another six isolates were resistant to 6-10 antibiotics, and 11 isolates were resistant to 11-18 antibiotics. The highest levels of resistance were found in isolates *Mesorhizobium*

sp. B13 (18 antibiotics) and *Pseudomonas* sp. B34 (15 antibiotics) (Table 3).

Antibiotic production

We tested 78 isolates (29% Gram-negative, 71% Gram-positive), from our culture collection for the production of growth inhibiting compounds. Thirty-six isolates (46%) inhibited the growth of at least one indicator strain. Twenty-three (29.5%) isolates inhibited the growth of two to nine indicator strains, while thirteen inhibited the growth of one indicator strain. The Gram-positive and Gram-negative isolates were equally effective in inhibiting the growth of indicator strains: 28 isolates inhibited the growth of Gram-positive indicator strains and 27 isolates inhibited the growth of Gram-negative indicator strains. As depicted in Table 4, most microbial mat isolates demonstrated unique patterns of antimicrobial activity. In general, most isolates suppressed the growth of *B. subtilis* (20 isolates, 25.6%), *S. aureus*

Fig. 3. Antibiotic resistance levels in Pajsarjeva jama isolates belonging to Gram-negative bacteria against various antibiotics.

Table 3. Range of antibiotic resistance to different antibiotics in Gram-negative isolates with respect to isolate genus.

Genus	Number of isolates	Number of resistances per isolate
<i>Agrobacterium</i>	6	9, 9, 12, 12, 12, 13
<i>Bosea</i>	1	5
<i>Lysobacter</i>	4	7, 7, 10, 12
<i>Mesorhizobium</i>	1	18
<i>Pseudomonas</i>	6	8, 14, 14, 14, 14, 15
<i>Sphingomonas</i>	1	4

MRSA (20 isolates, 25.6%) and *S. pseudointermedius* MRSP (18 isolates, 23.1%), demonstrating their potential as a tool in combating multi-resistant pathogenic bacteria. Furthermore, 15.4% of isolates inhibited the growth of ESBL *E. coli* Ž39, and 10% isolates inhibited the growth of *Salmonella enterica* subsp. Typhimurium and *Listeria monocytogenes*. The growth inhibition was lowest for indicator strains of *Klebsiella pneumoniae* and *Bacillus cereus* (inhibited by 0.1% of isolates). The three most active isolates

exhibited activity against seven and nine indicator strains, respectively, and corresponded to *Bacillus* sp. B1, *Pseudomonas* sp. AM82, and *Streptomyces* sp. AM91.

The effect of media choice on antibiotic production

We attempted to trigger 78 bacterial isolates to produce different secondary metabolites by incubating them on five different growth media that differed in nutrient composition. While these media differed in their ability to elicit production of antibiotics (Table 5), this difference was not statistically significant ($F = 1.09$; $P < 0.05$).

DISCUSSION

In this study, a bacterial culture collection originating from the microbial mats that grow on the walls of Pajsarjeva jama in Slovenia was screened for the production of antimicrobials and resistance to antibiotics. Growth of color distinct microbial mats,

Table 4. Antimicrobial activity of individual Pajsarjeva jama isolates against 10 selected pathogenic indicator strains. The isolates are grouped according to their taxonomic affiliation at genus level.

Taxonomic affiliation/ Gram stain	Isolate name	<i>E. coli</i> DH5	<i>E. coli</i> Ž39 ¹ (ESBL)	<i>S. enterica</i> s. Typhimurium TL 747	<i>K. pneumoniae</i> ATCC® BAA -1705 (CPE) ²	<i>K. pneumoniae</i> ATCC® BAA -1706	<i>B. cereus</i>	<i>B. subtilis</i>	<i>L. monocytogenes</i>	<i>S. aureus</i> (MRSA) ³	<i>S. pseudointermedius</i> (MRSP) ⁴	Total
<i>Agrobacterium</i> (G-)	B18	+				+		+		+		4
	B41			+	+			+	+	+	+	6
	B37	+	+					+	+	+		5
	Bx	+						+	+	+		4
<i>Aerococcus</i> (G+)	B60			+								1
<i>Bacillus</i> (G+)	B1	+	+		+	+	+	+	+	+	+	9
<i>Kocuria</i> (G+)	B35	+								+	+	3
<i>Lysobacter</i> (G-)	AM121				+							1
	B21	+						+	+	+	+	5
	B25		+							+	+	3
	B9		+							+	+	3
<i>Micrococcus</i> (G+)	B17									+		1
	B32										+	1
	B11									+		1
<i>Pseudomonas</i> (G-)	AM82		+	+	+		+	+		+	+	7
	B34						+	+	+	+	+	5
	B8					+	+	+		+	+	5
	AM71		+			+	+	+		+	+	6
<i>Rhodococcus</i> (G+)	AM78			+								1
	AM64							+				1
	AM68							+		+		2
	B36			+	+	+		+			+	5
	B27		+									1
	B20	+							+			2
<i>Sphingomonas</i> (G-)	B31		+					+	+	+	+	5
<i>Streptomyces</i> (G+)	30				+							1
	AM97							+			+	2
	PB4			+								1
	AM66	+	+					+		+	+	5
	AM84		+									1
	AM90							+			+	2
	AM72									+		1
	AM74			+								1
	AM91	+	+	+		+		+		+	+	7
	AM93		+		+	+	+	+				5
	AM108							+			+	2
	N° of isolates	9	12	8	7	7	6	20	8	20	18	
	% of all isolates	11.5	15.4	10.2	0.1	0.1	0.1	25.6	10.2	25.6	23.1	

Legend: ¹ESBL – extended spectrum beta-lactamase; ²CPE – carbapenem resistant enterobacterium; ³MRSA – methicillin-resistant *S. aureus*; ⁴MRSP – methicillin-resistant *S. pseudointermedius*

Table 5. Numbers of Pajsarjeva jama isolates showing antimicrobial activity with respect to growth medium.

Indicator strain	Number of isolates with antimicrobial activity				
	TSA	MHM	MG	LB	HA
<i>E. coli</i> DH5	3	2	5	2	2
<i>E. coli</i> Ž39 (ESBL)	7	4	4	2	3
<i>S. enterica</i> s. typhi TL 747	3	1	6	0	0
<i>K. pneumoniae</i> ATCC® BAA -1705 (CPE) ²	5	2	2	4	3
<i>K. pneumoniae</i> ATCC® BAA -1706	3	2	2	4	5
<i>B. cereus</i>	4	3	3	4	3
<i>B. subtilis</i>	8	17	8	10	16
<i>L. monocytogenes</i>	4	3	6	4	4
<i>S. aureus</i> (MRSA) ³	11	11	9	10	10
<i>S. pseudointermedius</i> (MRSA) ⁴	8	14	10	10	10

Legend: ¹ESBL – extended spectrum beta-lactamase; ²CPE – carbapenem resistant enterobacterium;

³MRSA – methicillin-resistant *S. aureus*; ⁴MRSP – methicillin-resistant *S. pseudointermedius*

white, yellow, grey or pink on the walls and sediments of karst caves is common in many caves. Cave mats can be observed in completely dark areas of the caves, often concentrated in the proximity of underground waters and sediments, but never in submerged areas. Past studies have shown that the dominant bacterial taxa in cave microbial mats corresponds to members of *Actinobacteria* and *Proteobacteria* as reported in other caves (Pašić et al., 2009; Northrup et al., 2011; Porca et al., 2012; Brag & Bergmann, 2019). However, since physicochemical conditions and nutrient availability differ in caves worldwide and even within a single cave, we were able to obtain culture collection of taxonomically novel bacterial isolates (Herzog-Velikonja et al., 2014).

We surveyed the antibiotic susceptibility of 69 culture collection isolates to 22 natural and synthetic antibiotics. The levels of antibiotic resistance were high, with majority of Gram-positive isolates being resistant to one to seventeen antibiotics and Gram-negative isolates being resistant to four to eighteen antibiotics. Certain amount of this resistance is intrinsic: we observed no resistance to antibiotic rifampin, which is used to treat infections with acid-fast microorganisms, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Likewise, high levels of resistance to antibiotic metronidazole (88% in Gram-positive and 90% in Gram-negative isolates) are likely a consequence of metronidazole acting primarily on anaerobic microorganisms indicating also low abundance of anaerobes in cave microbial mats. Other differences stem from different structure of Gram-negative cell wall and versatile resistance mechanisms in this group of bacteria. We measured comparatively less resistance to natural antibiotics than to synthetic antibiotics. This was somewhat surprising, as natural products are more likely to be present in the environment. Besides, in a study on Lechuguilla Cave, a pristine environment free from anthropogenic influences, the opposite trend was observed (Bhullar et al., 2012). All isolates were comparatively most resistant to modified β -lactams (resistance levels 30-80%), particularly to cephalosporines of second and third generation. Interestingly, low levels or resistance (<10%) were found even for cefepime, a recently introduced 4th generation cephalosporine. In

these terms, the results of our study are consistent with previous studies which have noted high levels of antibiotic resistance genes in cave environments (Pawlowski et al., 2016; Lavoie, 2017; Belyagoubi et al., 2018) and even in ancient cave microbes that had no anthropogenic contact for the past four million years (Bhullar et al., 2012).

Our culture collection contained isolates from genera whose members are well-known human and animal pathogens, often resistant to multiple antibiotics which makes treatment and patient recovery difficult. Examples are agents of nosocomial infections *Pseudomonas* and *Sphingomonas* (Obritsch et al., 2005; Göker et al., 2017), emerging pathogen *Kocuria* (Purty et al., 2013), opportunistic pathogen *Oerskovia* (Cruickshank et al., 1979) and honeybee pathogen *Paenibacillus* (Grady et al., 2016). In these terms we found establishing antibiotic resistance patterns important, as growing body of evidence suggests that non-pathogenic environmental organisms are a reservoir of resistance genes that have the potential to be transferred to pathogens (Riesenfeld et al., 2004; Allen et al., 2010).

Pseudomonas aeruginosa is the most important pathogen in the genus and displays resistance to a variety of antibiotics, including aminoglycosides, quinolones and β -lactams (Hancock & Speert, 2000). In our study, *Pseudomonas* members were also resistant to macrolides erythromycin (83% of *Pseudomonas* strains) and azithromycin (50% of *Pseudomonas* strain). All members of *Kocuria* are resistant to nitrofurantoin (Savini et al., 2010), which is consistent with our results, while resistance to other antibiotics varied among isolates. Similar to pathogenic isolates, our *Sphingomonas* isolate was resistant to 3rd generation cephalosporins cefotaxime and cefpodoxime (Bayram et al., 2013).

Most isolates in our culture collection belong to bacterial genera that are well known sources of bioactive compounds. Apart from members of genus *Streptomyces*, we obtained members of *Lysobacter*, a known prolific producer of bioactive peptides (Xie et al., 2012) and members of *Kocuria*, *Arthrobacter* and *Rhodococcus*, producers of compounds with antimicrobial, antifungal, and cytotoxic activities against human cells (Elsayed, 2017; Singh et al., 2017).

Previous studies on cave environments have noted antibiotic production (Yücel & Yamaç, 2010; Bhullar et al., 2012; Montano & Henderson., 2012; Tomova et al., 2013, Pawlowski et al., 2016; Riquelme et al., 2017) and resulted in a description of Xiakemycin A, a novel pyranonaphthoquinone antibiotics produced by a cave *Streptomyces* sp. (Jiang et al., 2015).

In this study almost half (46%) of tested isolates inhibited the growth of at least one indicator strain. The isolates were most active against multi-resistant *Bacillus* and *Staphylococcus* species (inhibited by 25% and 23% of tested isolates), *Klebsiella pneumoniae*, and *Listeria monocytogenes* (inhibited by 10% of tested isolates). These levels of inhibition were higher than those observed in a study on actinomycetes originating from a volcanic cave, where 26.5% of isolates inhibited *K. pneumoniae*, 2.0% inhibited *S. aureus*, and 1% inhibited ESBL *E. coli* (Cheeptham et al., 2013). The results of these two studies combined demonstrate the potential of cave microorganisms to suppress the growth of multi-resistant pathogens.

In our study, the three most bioactive isolates belonged to genera *Bacillus*, *Pseudomonas*, and *Streptomyces* and inhibited seven to nine indicator strains. Important levels of inhibition were also observed with *Lysobacter* and *Kocuria* members, which inhibited the growth of five indicator strains. The exact nature and mechanisms of growth-inhibiting compounds remains to be established in further studies.

In conclusion, this study suggests a high possibility of finding novel antimicrobial compounds in microbes that inhabit underground environments, while antimicrobial resistance patterns contribute to knowledge on environmental resistances and their presence in the environment.

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