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Isolation and identification of some antimicrobial producing bacteria from caves in Summan plateau, Saudi Arabia

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Abstract: *Caves in Kingdom of Saudi Arabia are not studied well in terms of microbial content. This study aimed to isolate and identify new bacterial genera that were antagonistic to some microbial pathogens from Mossy cave in Summan region. This cave located at the north of Riyadh. From 8 soil samples, 56 bacterial isolates were obtained on nutrient agar plates. The abilities of the isolates to antagonize with some pathogenic bacteria, yeasts and fungi were studied. The isolates AC30 and AC32 were the most antagonistic isolates to Staphylococcus aureus using agar well diffusion method, thus they were selected to apply further experiments. The two isolates were inoculated on different media and the best antagonistic effects were obtained using nutrient agar and sucrose nitrate media for isolate AC30 and AC32, respectively. The effect of different factors such as pH value, temperature and incubation period on the antimicrobial efficiency against microbes were determined. The best inhibitory activities of isolate AC30 were recorded in medium at pH 7 and incubation temperature at 37°C for 48 hrs. For isolate AC32, medium at pH 6 and incubation at temperature 35° C, for 48 hrs were the best conditions for inhibitory substance production. Finally, the two isolates were identified using morphological, physiological and biochemical characters. Their identification was confirmed using 16SrRNA as Paenibacillus polymyxa AC30 and Paenibacillus peoria AC32.*

Kay words*: Staphylococcus aureus, Mossy cave, Paenibacillus polymyxa, peoria, antimicrobial*

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I. Introduction

Antibiotics have been developed for more than 80 years but presently there is an evident decline on their effectiveness due to the development of resistance by pathogens. The arising of multi drug resistant pathogens has become a serious threat to public health. Microorganisms play a central role in nature as well as in drug discovery with a long track record as important sources of bioactive natural products revealing a wide array of molecules. Many approaches have been applied to solve this crisis, in the last decades the focus on extreme habitats as gain attention due to peculiar features and due to the presence of unique microbial populations inhabiting these ecosystems. Caves are considered extreme environments, usually oligotrophic, being in general devoid of light, and presenting stable conditions (temperature, humidity) throughout the year.

The emergence of multidrug resistant bacteria has placed a stress on health care systems and highlighted the need for new classes of antibiotics. The development of new antimicrobial agents, preferably naturally occurring ones with novel mechanisms of action, is an urgent medical need. Soil in particular is an intensively exploited ecological niche, the inhabitants of which produce many useful biologically active natural products, including clinically important antibiotics. However, the emergence of drug and multidrug-resistant pathogens necessitates a continuing search for new antimicrobial compounds with potent antipathogenic activity (Talbot, *et al*., 2006). Searching for previously unknown microbial strains is an effective approach for obtaining new biologically active substances (Fenical *et al.,* 1999). There is an urgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogens and to treat life-threatening diseases such as cancer (Fischbach and Walsh, 2009, Payne, *et al*., 2007). The need for less toxic, more potent antibiotics, which overcome the resistance exhibited against the existing antibiotics, is increased (Dhanasekaran, *et al.,* 2009).

Species belonging to the *Paenibacillus* genus were previously re-classified under the genus *Bacillus*, based on morphological characteristics. In nature, the habitat of *Paenibacillus* spp. is mainly soil. Due to their multiple beneficial effects, *Paenibacillus* spp. have implications for agriculture, environmental remediation, and even human and animal health (Jeong *et al*., 2019).

A wide variety of bacteria produce lipopeptides, however *Bacillus* and *Paenibacillus* spp. in particular have yielded several potent antimicrobial lipopeptides. Many of the lipopeptides produced by these bacteria have been known for decades and represent a potential gold mine of antibiotic candidates. This list includes the polymyxins, octapeptins, polypeptins, iturins, surfactins, fengycins, fusaricidins, and tridecaptins, as well as some novel examples, including the kurstakins. These lipopeptides have a wide variety of activities, ranging from antibacterial and antifungal, to anticancer and antiviral. Emphasis has been placed on their antimicrobial

activities, as well other potential applications for this interesting class of substances (Cochrane and Vederas, 2016).

The microbial life in some of Saudi Arabia caves was not well studied. There is a very limited studies to identify the bacteria with antimicrobial activities in the Mossy cave. The research aims to isolate antimicrobial producing bacteria from caves in Summan area northeast of Riyadh, Saudi Arabia and identifies them in a molecular level.

II. Materials And Methods

Soil sample collection and treatment:

Eight samples of soil were collected from Mossy cave in Summan plateau northeast Al-Riyadh, Kingdom of Saudi Arabia (26°27'33.2″N, 47°14'03.3″E,). The soil samples were used for bacterial isolation on Nutrient agar (NA) using serial dilution method.

Purification and preservation of the isolates:

The pure bacterial colonies were selected to be isolated and purified on NA medium and preserved at 4°C until used. For long-time preservation, stock of 15% sterile glycerol in a small vial were inoculated with a single colony of the bacterial strain and preserved at -80°C.

Test organisms

Staphylococcus aureus, *Salmonella* sp., *Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* sp., *Acinetobacter* sp. and *Candida* were used as a test organisms and were obtained from Maternity and Children's Hospital in Jeddah. The isolates *Penicillium* sp., *Fusarium* sp., and *Aspergillus* sp. were obtained from Microbiology Lab., in king Abdulaziz University.

Antimicrobial activity of the isolated bacteria:

Isolates of bacteria were purified and tested for antagonistic activity against the tested bacteria and fungi. The antimicrobial activity of the isolated bacteria was determined using agar well diffusion methods and Mueller-Hinton agar medium (Muhammad *et al.,* 2009). The diameter of inhibition zones was measured after incubation for 24 hrs. at 37˚C for the bacteria and yeast while fungi were incubated at 25°C for 4days.

Bacterial identification

The isolated bacteria were identified based on Bergey's Manual of Systematic Bacteriology (De Vos *et al*., 2009). After testing the bacteria by the conventional methods, the two selected bacteria were sent to Macrogen for identification by 16S rRNA sequence analysis; Inc., South Korea public biotechnology company.

Effect of different media on antimicrobial activity of isolates AC30 and AC32:

Several broth media were used to test the ability of the active of isolate to produce inhibitory bioactive compounds against the *Staphylococcus aureus*. A comparative study was carried out on the production of bioactive compounds in Nutrient broth, Glucose- peptone medium , Glucose- asparagine, Rich medium and Sucrose – nitrate medium (Agwa *et al*., 2000). Rich medium, Glucose- asparagine medium, Glucose- peptone medium, Nutrient broth and Sucrose- nitrate medium. Fifty ml of these different media were dispensed in 250 ml flasks. After sterilization, the flasks were inoculated with 2 ml of precultures and incubated using shaking incubator (120 rpm and 37°C for 48 hrs.). The cell-free filtrate was tested for antimicrobial activity against *Staphylococcus aureus*.

3.4.6 Effect of different initial pH value on antimicrobial activity of isolates AC30 and AC32:

Fifty ml of culture broth witch is Nutrient broth for isolate AC30 and Sucrose nitrate broth for AC32, were dispensed in 250 ml flasks and adjusted to the required pH value at 5.0, 6.0, 7.0. 8.0 and 9.0 using either 1 N NaOH or l N HCl. After sterilization, the flasks were inoculated with 2 ml of precultures and incubated using shaking incubator (120 rpm and 37˚C for 48 hrs. The cell-free filtrate was tested for antimicrobial activity against *Staphylococcus aureus*.

Effect of incubation temperature on antimicrobial activity of isolates AC30 and AC32:

Erlenmeyer Flasks (250 ml capacity) each containing fifty ml of culture broth witch is Nutrient broth for AC30 and Sucrose nitrate broth for AC32, at pH 7 (the best pH value determined from the previous experiment for isolate AC30) and pH 6 for isolate AC32 were sterilized and inoculated with 2 ml of precultures. The flasks were incubated in a shaker (120 rpm for 48 hrs.) at different incubation temperature including 28, 30, 35, 37 and 40˚C. Cells were harvested as previously mentioned and the cell free filtrate was used to determine its antagonistic activity against *Staphylococcus aureus*.

Effect of incubation time on antimicrobial activity of isolates AC30 and AC32 :

Fifty ml of the media were dispensed in 250 ml flasks. After sterilization, the flasks were inoculated with 2 ml of pre cultures and incubated for 24, 48 and 72 hrs. using shaking incubator (120 rpm) at 37°C for isolate AC30 and 35°C for isolate AC32. The cell-free filtrate was tested for antimicrobial activity against *Staphylococcus aureus*.

III. Result

The antimicrobial activities of the two selected isolates

Among the 56 isolated bacteria, two isolates assigned isolates AC30 and AC32 which were obtained from soil samples, showed the greatest inhibitory activity against some pathogenic bacteria, fungi and yeast (Figure 1). The tested microbes were including bacteria (*Staphylococcus aureus*, *Salmonella* sp., *Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* sp., *Acinetobacter* sp.), fungi (*Penicillium* sp., *Fusarium* sp., and *Aspergillus* sp. and *Candida* sp.).

Both isolates were highly active against *Staph. aureus* but *Shigella* sp. was not affected by any of the tested isolates of bacteria. Isolate AC30 was less active against *Escherichia coli* and *Pseudomonas aeruginosa* than against other pathogenic bacteria, while isolate AC32 was less active against *Klebsiella* sp. and *Salmonella* sp. than against other pathogenic bacteria (Table 1). The two isolates were tested as antifungal agents and the highest activities for the two isolates were against *Candida* sp., *Penicillium* sp., *Fusarium* sp., and *Aspergillus* sp. and the inhibition zone were ranged from 11-17 mm (Table 2).

Table 1. Antagonistic of samples AC30 and AC32 against tested bacteria

Bacteria	AC30	AC32
Staphylococcus aureus	17± 0.67	23± 2.90
***Salmonella* sp.**	13± 0.67	11± 0.67
***Klepsella* sp.**	15± 1.15	10± 0.88
Escherichia coli	13± 0.67	15± 2
Pseudomonas aeruginosa	10± 0.88	13± 0.88
***Shigella* sp.**	Not detected	Not detected
***Acinetobacter* sp.**	15± 0.67	17± 0.67

Table 2. Antagonistic activity of isolates AC30 and AC32 against tested fungi and yeast

Fungi and yeast	AC30	AC32
***Penicillium* sp.**	15± 4.80	13± 1.33
***Fusarium* sp.**	13± 0.67	11± 1.15
***Aspergillus* sp.**	13± 0.67	13± 1.76
***Candida* sp.**	15± 1.76	17± 1.33

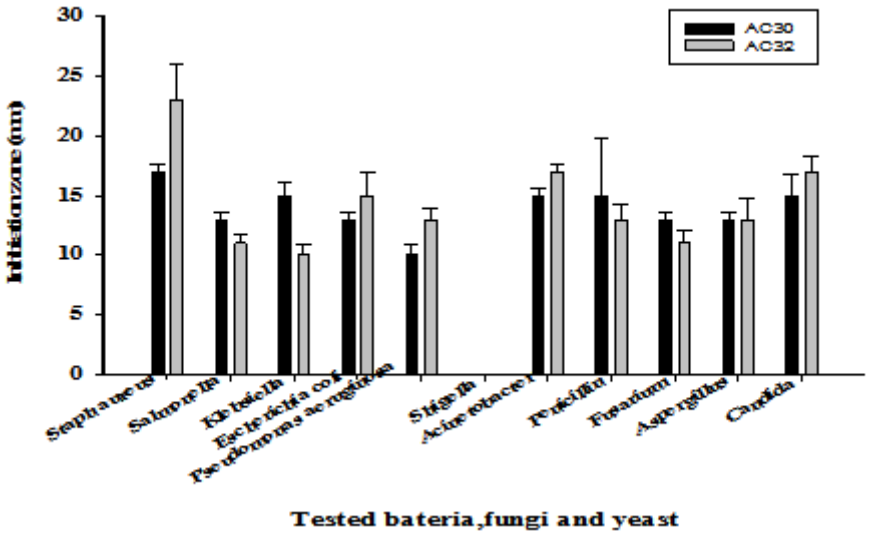

Figure 1. Inhibition zone diameter (mm) obtained by the two bacterial isolates against the tested bacteria & fungi

Factors affecting antibiotic production by the two selected isolates

Effect of different media, Nutrient broth, Glucose- peptone medium, Glucose- asparagine, Rich medium and Sucrose – nitrate medium on antimicrobial production was studied. The results of different media showing that the best media for antimicrobial production for isolate AC30 is Nutrient broth and for isolate AC32 is Sucrose-nitrate medium (Figure 2).

Also, the isolate AC30 showed the highest antimicrobial production at pH7 while isolate AC30 prefer medium with pH6 (Figure 3). For antimicrobial production, 37℃ was the best temperature for isolate AC30 and the 35℃ was the best temperature for isolate AC32 (Figure 4). For antimicrobial production, 48 hours was the best incubation period for isolate AC30 and AC32 (Figure 5).

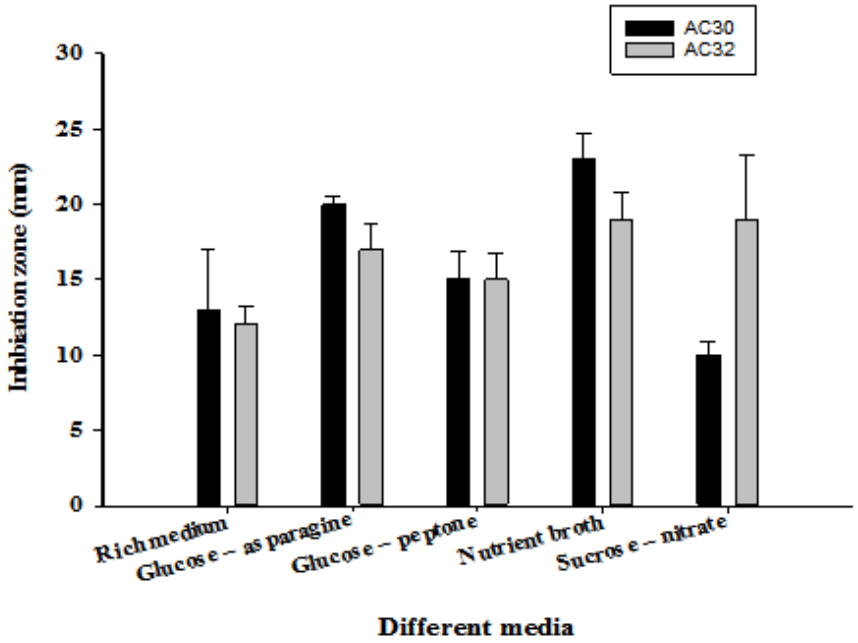

Figure 2. Effect of different media on antimicrobial production by the two selected isolates

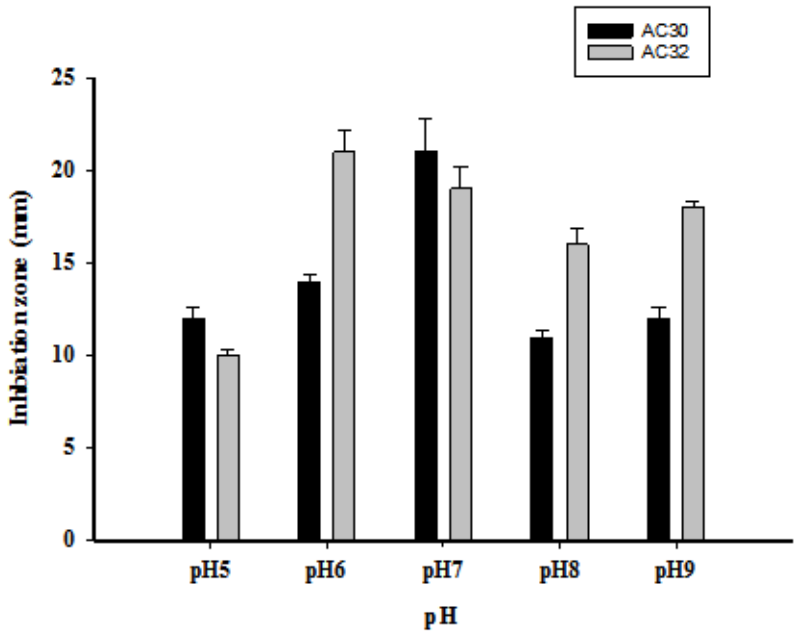

Figure 3. Effect of different pH on antimicrobial production by the two selected isolates

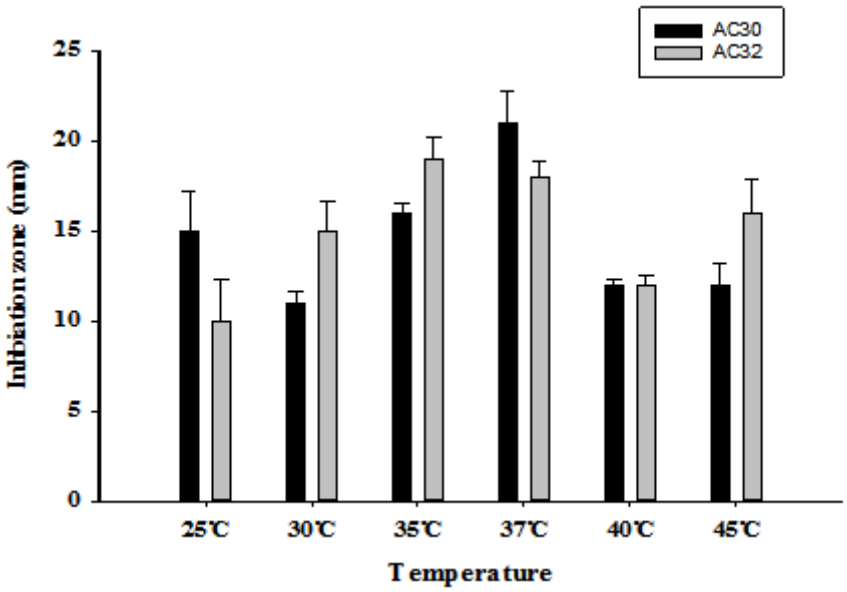

Figure 4. Effect of different temperature on antimicrobial production by the two selected isolates

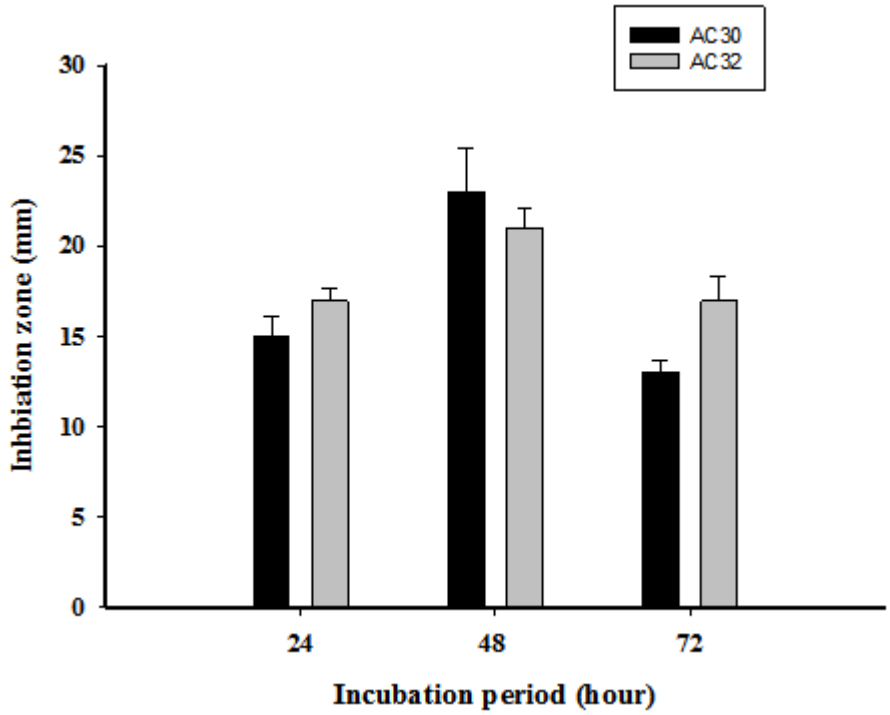

Figure 5. Effect of different incubation period on antimicrobial production by the two selected isolates

Identification and characterizations of isolates AC30 and AC32:

The morphological characteristics of isolates AC30 and AC32 were specified as Gram-positive, aerobic, rod-shaped, endospore-forming bacteria. Colonies are a smooth and translucent with white color (Table 3). The two isolates were belong to genus *Paenobacillus*. They were identified as *Paenibacillus polymyxa* and *Paenibacillus peoriae* using 16S rRNA (Table 4). The phylogenic tree of the two identified bacteria was shown in Figure 6.

Table 3. Biochemical and physiological characteristics of isolates *Paenibacillus polymyxa* AC30 and *Paenibacillus peoriae* AC32

Characteristic	***Paenibacillus polymyxa***	***Paenibacillus peoriae***
Gram stain	+	+
Cell shape	rod	rod
Motility	+	+
Endospore forming	+	+
Oxygen requirement	aerobic	aerobic
Catalase	+	+
Oxidase	–	–
Urease	–	–
Starch	+	+
Gelatin hydrolysis	+	+
Indole	-	-

+Positive results, -: negative results

Table 4. Phylogenetic identification of two soil bacteria isolated from Summan cave and theirs most related groups

NO	Samples	Closest matches identification	NBCL (Accession number)	Sequence identity
1	Soil	*Paenibacillus polymyxa*	NR_112641.1	99%
2	Soil	*Paenibacillus peoriae*	NR_117743.1	99%

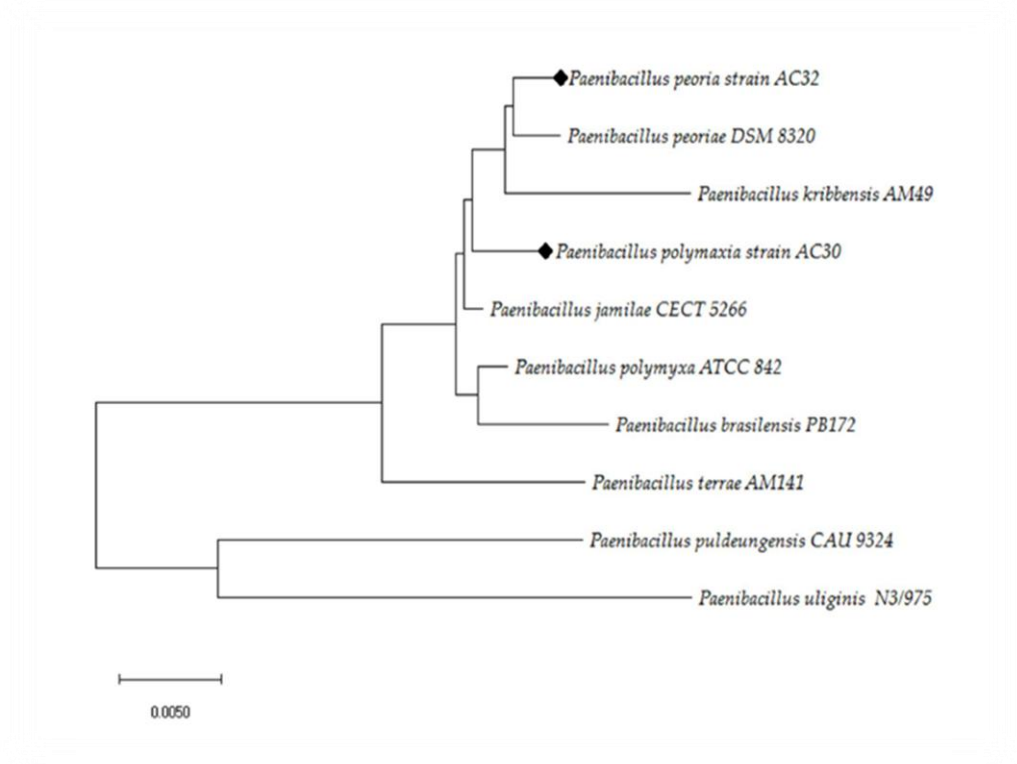

Figure 6. phylogenic tree of the two identified bacteria

IV. Discussion

The study of cave microbiomes has been at the center of biologists' attention for the last few decades. Both the microbial diversity in cave habitats and the potential for the production of unique primary and secondary metabolites were differ from those found in other extreme habitats (Ghosh *et al.*, 2017). The tested

bacteria was isolated on Nutrient agar by serial dilution method (Gowsalya *et al.,* 2014), then the obtained bacteria were purified and preserved. Agar well diffusion assay was used to check the production of antimicrobial metabolites by the two tested bacteria. Test cultures of pathogenic bacteria, yeast and fungi were grown to 0.5 McFarland standard of optical density and spread on Muller Hinton agar plates. With the help of sterilized borer, wells were made in the agar plates and culture filtrate of the producing organism was poured in the well and incubated for 24 hrs, the zone of inhibition was measured for the activity of antimicrobial compounds (Muhammad *et al.,* 2009).

Identification and characterizations of isolates AC30 and AC32 was carried out according to morphological, physiological and biochemical characterizations as described by Lihan *et al.,* (2014). They were identified on the bases of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2000). The morphological characteristics of isolate were observed by Gram and spore staining. Analyses for the biochemical properties of isolate included catalase, oxidase, and urease reactions, gelatin liquefaction, starch hydrolysis, and indole production (He *et al.,* 2007). The two isolates belong to genus *Bacillus*. Identification of bacteria isolate was confirmed by 16S rRNA sequence analysis (Macrogen, http://www.macrogen.co.kr, Seoul, South Korea) (Kim *et al.,* 2018). Similarly, analysis of the average nucleotide identity across complete genome sequences of *Paenibacillus polymyxa* strains (E681 and SC2) were determined and draft sequences of *P. polymyxa* ATCC 842T and *Paenibacillus peoriae* KCTC 3763T revealed that *P. peoriae* KCTC 3763T is closer to E681. The two isolates were producer to more than one antibiotic (Richter and Rossello-Mora, 2009).

Analysis of antimicrobial activity of bacteria isolated from Cerâmica Cave, located in the Sicó karst massif, central Portugal was determined. Microorganism acquisition was performed through a variety of physical and chemical treatments: dry-heat, wet-heat, dry-heat plus phenol (1,5%), phenol (1,5%), microwave irradiation, rehydration centrifugation, and no-treatment, along with three media were used (Gouveia *et al*., 2019). A primary antimicrobial activity screening, through cross streak method, was performed in 28 isolates, using two different incubation periods and different target organisms (*Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Aeromonas salmonicida, Salmonella typhimurium* and *Enterococcus faecalis*). A total of 7 isolates presented antagonistic activity against *B. cereus, P. aeruginosa, E. coli, A. salmonicida, S. typhimurium* and *S. aureus*. Their study proved the ability of cave microorganisms to inhibit both Gram positive and Gram negative pathogenic strains, highlighting the potential of caves microbiome to be one of the answers to solve the current global crisis of multi drug resistant pathogens (Gouveia *et al*., 2019). A subterranean limestone cave, Manao-Pee, was investigated for bacterial diversity and potential secondary metabolites production (Wiseschart *et al*., 2017). Comparative 16S rRNA analysis revealed that cave soil was highly dominated by Actinobacteria; whereas, Proteobacteria was highly abundant outside the cave (Amasha, 2018).

The exploration of antibiotic resistance in the Paenibacillaceae prompted by the discovery of an ancient intrinsic resistome in *Paenibacillus* sp. LC231, recovered from the Lechuguilla cave environment (Pawlowski *et al*., 2018). Recently, there has been an increasing demand for new molecules with bioactivity as a result of the increasing number of multi drug resistant bacteria and also of the need to find treatment for various diseases. Thus, to fill this urging need there has been a great investment in developing new techniques to speed up and increase the possibility of finding new molecules with therapeutic potential. In conclusion, studying caves in Saudi Arabia is very promising and their soils are filled with new bacterial genera with antimicrobial activities.

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Conflict of Interest Authors would hereby like to declare that there is no conflict of interests that could possibly arise

Reference:

[1]. Agwa, A., Aly, M. M., Bonaly, R (2000): Isolation and characterization of two *Streptomyces* species produced non polyenic antifungal agents. J. Union Arab Biol. Vol.7 B, pp.: 62- 84.

[2]. Amasha R H (2017). Microbial diversity and phylogenetic studies of some microbes obtained from unexplored caves of Saudi Arabia. Journal of Experimental Biology and Agricultural Sciences, April - 2018; Volume – 6(2) page 342 – 351.

[3]. Cochrane, S.A. and Vederas, J.C. (2016) Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. Medicinal Research Reviews,36(1) p: 4-31.

[4]. De Vos P, Wolfgang Ludwig, Karl-Heinz Schleifer And William B. Whitman (2009). Family IV. Paenibacillaceae fam. nov., Paenibacillus type genus of the family; suff. -aceae ending. In: Bergey's Manual of Systematic Bacteriology. https://www.springer.com/gp/.

[5]. Dhanasekaran D, Thajuddin N, Panneerselvam A., 2009. Distribution and ecobiology of antagonistic streptomycetes from agriculture and coastal soil in Tamilnadu, India. J Cult Collection;V 6: P 10-20

[6]. Fenical W, Baden D, Burg M, de Goyet CV, Grimes JD, Katz M, *et al*., (1999). Marine derived pharmaceuticals and related bioactive compounds. In: Fenical W, editor. From monsoons to microbes: understanding the ocean's role in human health. Washington DC: National Academies Press.
[7]. Fischbach MA, Walsh CT., (2009). Antibiotics for emerging pathogens Science; V325: P 1089-93.
[8]. Ghosh, S., Kuisiene, N. and Cheeptham, N., 2017. The cave microbiome as a source for drug discovery: Reality or pipe dream?. *Biochemical pharmacology*, 134, pp.18-34.
[9]. Gouveia, A., Reboleira, A.S.P., Gonçalves, F. and Marques, S., (2018). Caves as a source of new antimicrobial agents: the case study of antibacterial activity from microorganisms inhabiting Cerâmica Cave, Portugal, 19: 219-229.
[10]. Gowsalya, A., Ponnusami, V. and Sugumaran, K.R., (2014). Isolation of bacteria from soil sample for exo-polysaccharide production. *International Journal of ChemTech Research*, *6*(5), pp.2925-2928
[11]. He, Z., Kisla, D., Zhang, L., Yuan, C., Green-Church, K.B. and Yousef, A.E., (2007). Isolation and identification of a Paenibacillus polymyxa strain that coproduces a novel lantibiotic and polymyxin. *Applied and Environmental Microbiology*, *73*(1), pp.168-178.
[12]. Holt J. G., NoelR. Krieg, Peter H.A. Sneath, James T. Staley, Stanley T. Williams. (2000) In: Bergey's manual of determinative bacteriology. &Wilkins,. Baltimore : Lippincott, Williams, p, 787.
[13]. Jeong H, Choi SK, Ryu CM, Park SH. (2019). Chronicle of a Soil Bacterium: *Paenibacillus polymyxa* E681 as a Tiny Guardian of Plant and Human Health. *Front Microbiol*.;10:467.
[14]. Kim, Y., Kim, H., Beuchat, L.R. and Ryu, J.H., (2018). Development of non-pathogenic bacterial biofilms on the surface of stainless steel which are inhibitory to *Salmonella enterica*. *Food microbiology*, 69, pp.136-142.
[15]. Lihan, S., Lin, C.S., Ahmad, I., Sinang, F.M., Hua, N.K. and Sallehin, A.A., (2014). Antimicrobial producing microbes isolated from soil samples collected from Nanga Merit Forest in Sarawak, Malaysian Borneo. *European Journal of Experimental Biology*, *4*(1), pp.494-501.
[16]. Muhammad, S.A., Ahmad, S. and Hameed, A., (2009). Antibiotic Production By Thermophilic *Bacillus* Specie Sat-4. *Pakistan journal of pharmaceutical sciences*, *22*(3).
[17]. Pawlowski, A.C., Westman, E.L., Koteva, K., Waglechner, N. and Wright, G.D., (2018). The complex resistomes of Paenibacillaceae reflect diverse antibiotic chemical ecologies. *The ISME journal*, *12*(3), p.885.
[18]. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL., (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov*; V6: P 29-40.
[19]. Richter, M. and Rosselló-Móra, R., (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the National Academy of Sciences*, 106 (45), pp.19126-19131.
[20]. Talbot, G.H., Bradley, J., Edwards Jr, J.E., Gilbert, D., Scheld, M. and Bartlett, J.G., (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clinical infectious diseases*, 42(5), pp.657-668.
[21]. Wiseschart A, Mhuanthong W, Thongkam P, Tangphatsornruang Sithichoke, Chantasingh D & Kusol Pootanakit (2017). Bacterial Diversity and Phylogenetic Analysis of Type II Polyketide Synthase Gene from Manao-Pee Cave, Thailand, Geomicrobiology Journal, 35:6, 518-527

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