EVALUATION OF ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES OF ACTINOBACTERIA ISOLATED FROM THE SALINE LAGOONS OF NORTHWEST PERU.

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Abstract

The unexplored saline lagoons of the north of Peru harbor a rich microbiome, due to reported studies of different extreme environments around the world. In these regions, there are several ecosystems and microhabitats not yet explored, and little is known about the diversity of actinobacteria and other microorganisms. We suggest that the endemic bacteria present in this extreme environment could be source of active molecules with anticancer, antimicrobial, antiparasitic properties. Using phenotypic and genotypic characterization techniques including the 16S rRNA were identified into the genera *Streptomyces* 39 (78%), *Pseudonocardia* 3 (6%), *Staphylococcus* 4 (8%), *Bacillus* 2 (4%), and *Pseudomonas* 2 (4%). All isolated bacteria for the genotypic data were preliminarily identified. Actinobacteria strains were found dominantly in both sites (Lagoon1-3 = 16 isolates and lagoon 4 = 12 isolates). Phylogenetic analysis revealed that 28 isolates were exclusively affiliated to eleven different clusters of Actinobacteria of the major genus *Streptomyces*. Three *Streptomyces* sp. strains M-92, B-146, and B-81, were tested for antibacterial and antiproliferative activities. The results showed antiproliferative activities against three tumor cell lines, U251 glioma; MCF7 breast; NCI-H460 lung non-small type of cells, and the antibacterial activity to *Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 10536, and *Acinetobacter baumanni* AC-972 which is resistant to multiple drugs. The promising results belong to *Streptomyces* sp. B-

81 strain in the R2A medium using a doxorubicin with control positive, the best result was from the latter (TGI = 0,57 µg/mL) for glioma; NCI-H460 lung of type non-small cells (TGI = 0,61 µg/mL), and breast cancer (TGI =0,80 µg/mL), this strain was selected to be fractionated because it had better antiproliferative and antibacterial activity, and its fractions were evaluated concerning antiproliferative activity against nine types of tumor cells and one non-tumor. The methanolic fraction showed a better result in the antiproliferative activity and was able to inhibit U251 (glioma) (TGI = 38.3 µg/mL), OVCAR-03 (ovary) (TGI = 62.1 µg/mL), and K562 (leukemia) (TGI = 81.5 µg/mL). The methanol 50% - acetate 50% fraction (Fraction 4) inhibited U251 (glioma) (TGI = 73.5 µg/mL) and UACC-62 (melanoma) (TGI = 89.4 µg/mL). Moreover, the UHPLC-MS/MS data and molecular networking of *Streptomyces sp*. B-81 isolate extract revealed the production cholic acid, Lobophorin A, Lobophorin B, Lobophorin E, Lobophorin K and compound 6. Extremophilic environments such as the Mórrope and Bayovar Salt Flats are promising sources of new bacteria with promising pharmaceutical potential; These compounds could be useful to treat various infectious diseases or even some type of cancer.

Keywords: *Streptomyces* sp. B-81; saline lagoons; Morrope salt flats; Bayovar salt flats; antimicrobial activity; antiproliferative activity.

1. Introduction

Perú has extreme environments such as the salt marshes located on the coast, center, and south of the country. In these regions, there are several ecosystems and microhabitats, whose studies are still restricted and report the diversity of bacteria and other organisms (Caceres

and Legendre, 2009; Flores and Pisfil, 2014; Leon et al. 2016). Among other microorganisms, the members of the phylum Actinobacteria can be found in all kinds of extreme environments. *Micromonospora*, *Actinomadura*, and *Nocardiopsis* were reported from saline soils of the ephemeral salty lakes in Buryatiya (Lubsanova et al. 2014). *Streptomyces*, *Nocardiopsis*, and *Nocardioides* were isolated from the Regions Western Ghats of India (Siddhart et al. 2020). Concurrent to this, there is still little information documented on isolated substances and their biological activities of the microorganisms isolated from saline environments. *Micromonospora*, *Streptomyces*, *Salinispora*, and *Dietzia* were isolated from the coastal zone of the geographically remote young volcanic Easter Island Chile. Besides, actinobacterial halophilic and halotolerant strains show heterogeneous physiological characteristics among different genera because these bacteria have the ability to synthesize secondary metabolites to cope with conditions of high salinity and the different temperatures present in their environments (Binayke et al. 2018; Oueriaghli et al. 2018; Boyadzhieva et al. 2018). These extreme conditions favor the development of metabolically competitiveness to the production enzymes, which can help the bacterial population to adapt to high salinity (Kim et al. 2019). Furthermore, these microorganisms can also perform essential processes such as carbon cycle, metal transfer, and the removal of organic pollutants to higher trophic levels (Fiedler et al. 2005; Blunt et al. 2007). In addition, this microbial group presents a unique ability to produce new products, mainly antibiotics (Berdy, 2005; Strohl, 2004).

The search for these microorganisms has been mainly related to the production of antibiotics and antitumor substances (Newman and Cragg, 2007). There are about 22.500 biologically active substances obtained from microorganisms, 45% of which are represented by

actinomycetes, with *Streptomyces* being the producer of 70% of them (Hayakawa et al. 2007 and Valliappan et al. 2014). According to Lam (2006), there are new actinobacteria from habitats not yet explored or little explored to be the source of new bioactive secondary metabolites.

The bioguided study strategy helps us find a wide variety of microorganisms that have biotechnological potential (Maldonado et al. 2005). This study reports the isolation and phylogenetic analysis of a collection of bacterial isolates from the saline lagoons from the Northern Peru, and their potential as producers of secondary metabolites with antimicrobial and antiproliferative activities.

2. Materials and methods

2.1 Site description and sampling

Sample collection at Mórrope saline lagoons was carried out in December 2012 the zone "1", in January 2013 the zone "2", and in July 2014 the zone "3", while samples were collected at Bayovar saline lagoons in March 2015 the zone "4" (Figure 1). The present work did not need permits from a competent authority, since it does not include any protected species, because the standard is currently in the process of being implemented to achieve its registration, likewise the sampling areas are lagoons that due to their nature are in the process of adjudication to the competent governmental organism through the Peruvian Ministry of the Environment.

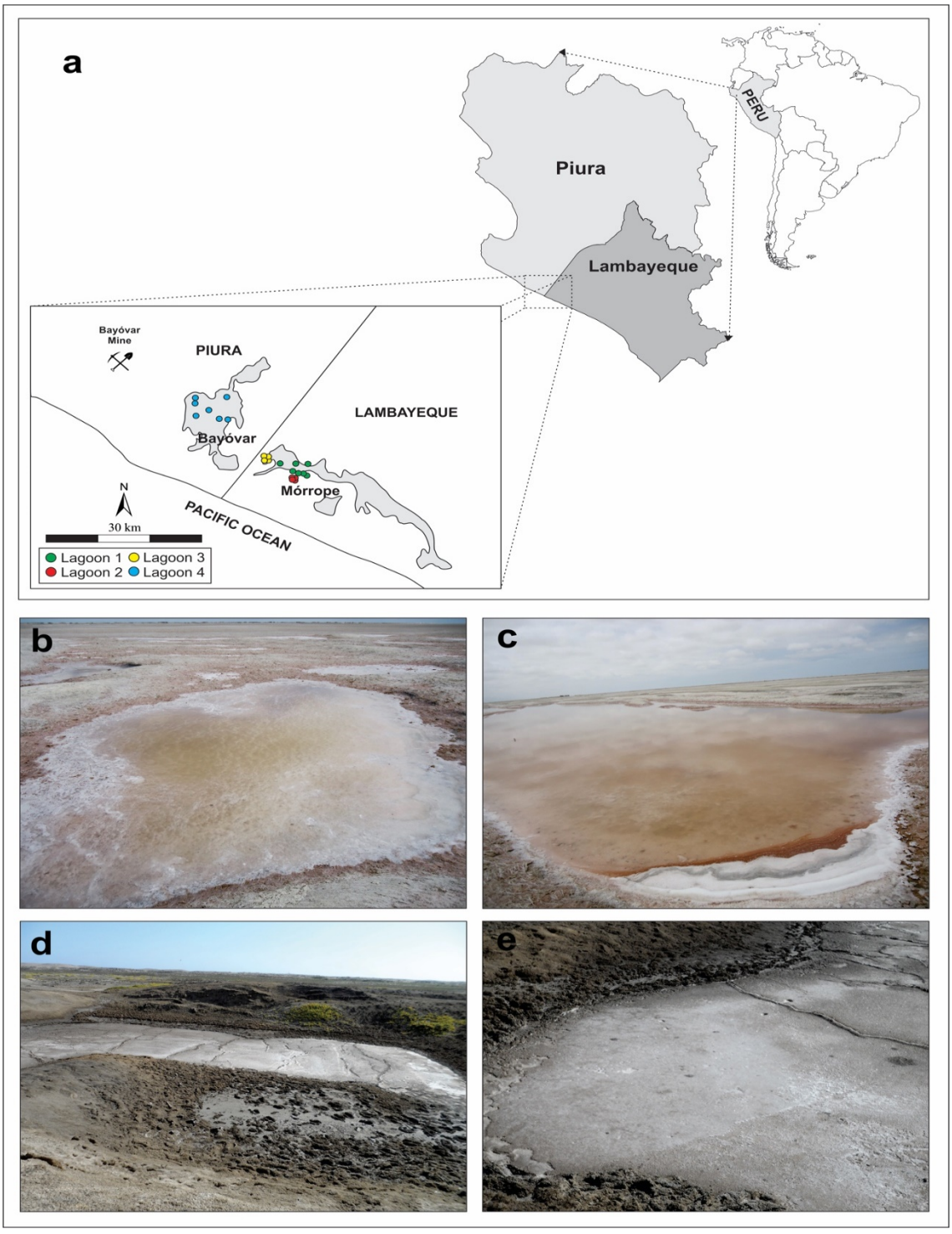


Fig 1. a = Geographic location of sampling sites; b and c = lagoons 1, 2 and 3 (State Mórrope); and e = lagoon 4 (State Bayovar).

116 The sources and places from which samples were obtained are detailed in Table 1. Samples
117 were collected aseptically, placed in sterile plastic bags, and kept refrigerated at 4 °C for
118 further processing in the Laboratory of Bacteriology the Regional Hospital of Lambayeque,
119 a flowchart depicting the methodological strategy adopted in this work is shown in Figure 2.
120

121 **Table 1. Data related to saline lagoons samples.**

Mórrope lagoons				
Collection zone	**Sample type**	**Geographic location**		
Zone 1 (December 2012)	A; AES	6°10S;80°35W (3 samples x triplicate)	6°11S;80°37W (3 samples x triplicate)	6°9S;80°39W (3 samples x triplicate)
Zone 2 (January 2013)	AES; SE	6°19S;80°28W (3 samples x triplicate)	6°17S;80°26W (3 samples x triplicate)	6°18S;80°27W (3 samples x triplicate)
Zone 3 (December 2014)	A; SE	6°08S;80°50W (3 samples x triplicate)	6°08S;80°51W (3 samples x triplicate)	6°08S;80°40W (3 samples x triplicate)
Bayovar lagoons				
Zone 4 (March 2015)	A; AES; SE	6°23S;80°66W (3 samples x triplicate)	6°25S;80°26W (3 samples x triplicate)	6°23S;80°78W (3 samples x triplicate)

122 A= (saline water), AES= (water and sediment), SE= (sediment).

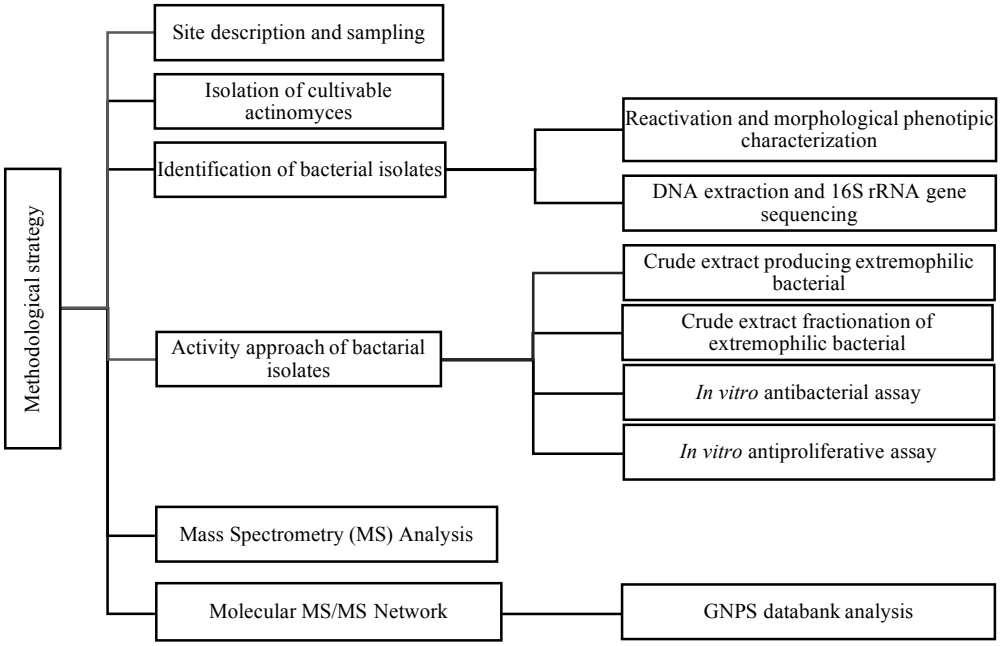


Fig. 2. Flowchart depicting the methodological strategy adopted in this study.

2.2 Isolation of cultivable actinomyces

An aliquot of 10.0 mL of saline lagoons sample was transferred to an Erlenmeyer containing 10.0 mL of salt broth (0.6% yeast extract, 2% glucose, 5% peptone, 3% meat extract supplemented with chloramphenicol and 1% fluconazole at pH 7.0). All Erlenmeyer were homogenized and maintained at 50 °C in a water bath for 60 min to reduce the pollutant load (Pisano et al. 1989 and Takizawa et al. 1993). Then, they were incubated at 28 °C for 7 to 30 days under aerobic conditions; after, the microorganisms were isolated in the media: modified saline agar (1.0 g yeast extract, 5.0 g peptone, and 15.0 g of agar at pH 7.6 ± 0.2) and trypticase soy agar (Bacto™) supplemented with saline water. The isolates were preserved in 20% glycerol and kept at -80 °C cryopreserved. The isolates were reactivated

and stored in a refrigerated chamber at -20 ° C to the transport to the Microbial Resources Division of the Pluridisciplinary Center for Chemical, Biological and Agricultural Research (CPQBA) in August 2015.

2.3 Identification of bacterial isolates

2.3.1 Reactivation and morphological phenotypic characterization of bacterial strains

The reactivation of the isolates was carried out in R2A broth (Difco ref. 234000), supplemented with artificial seawater (ASW) NaCl 5% (0.1 g potassium bromide, 23.48 g sodium chloride, 10.61 g of magnesium chloride, 1.47 g of calcium chloride, 0.66 g of potassium chloride, 0.04 g of strontium chloride, 3.92 g of sodium sulfate, 0.19 g of sodium bicarbonate, 0.03 g of boric acid all in 1 L of distilled water) at 28 °C for 28 days, the colonies were observed under a microscope and stereoscope for macro morphological characterization and after Gram staining, the cells were observed under an optical microscope. The bacteria were preserved in 20% glycerol and kept at -80 °C.

2.3.2 DNA extraction and 16S rRNA gene sequencing

An isolated colony was used for extraction of genomic DNA, according to the method described by Pospiech and Neuman, 1995, with some modifications. Amplification of the 16S ribosomal RNA gene was done by means of a polymerase chain reaction (PCR) using the pair of primers p10f (5’ GAG TTT GAT CCT GGC TCA G 3’) and p1401r (5’ CGG TGT GTA CAA GGC CCG GGA ACG 3’) or p1492r (5’- ACC TTG TTA CGA CTT 3’) (Lane et al. 1985) homologous to conserved regions of the bacterial 16S ribosomal RNA

gene. Conditions used in the amplification reaction were: 2.5 µL of 10X buffer (without Mg^{+2}), 0.75 µL of $MgCl_2$ (2.5 mM), 0.2 µL dNTP (2.5 mM each), 0.5 µL of each primer (20 µM), 0.2 µL of Taq DNA polymerase (Invitrogen) and 5.0 µL of the DNA template (50-100 ng/ µL) and deionized and sterilized water by filling the volume to 25 µL of reaction. The PCR amplification was an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, 3 min at 72°C for extension and 3 min at 72°C for final extension in an Eppendorf thermal cycler. The obtained products were analyzed and visualized in agarose gel electrophoresis, purified with mini columns (GFX PCR DNA & gel band purification kit, GE Healthcare), and sequenced in ABI3500XL Series automatic sequencer (Applied Biosystems). Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's specifications.

Partial sequences of the 16S ribosomal RNA gene obtained from each isolate were assembled into a contig and then compared to the sequences of organisms represented in EZBioCloud 16S Database (https://www.ezbiocloud.net/) using the “Identify” service (Yoon et al. 2017), and species assignment were based on closest hits (Jeon et al. 2014). 16S ribosomal RNA gene sequences retrieved from the database and related to the unknown organism gene were selected for alignment in the Clustal X program (Thompson et al. 1997), and phylogenetic analyzes were performed using the Mega version 6.0 program (Tamura et al. 2013). The evolutionary distance matrix was calculated with the model of Kimura-2 parameters (Kimura, 1980), and the phylogenetic tree constructed from the evolutionary distances calculated by the Neighbor-Joining method (Saitou and Nei, 1987), with bootstrap values from 1000 resampling.

183

184 **2.4 Activity approach of bacterial isolates**

185 **2.4.1 Crude extract producing extremophilic bacterial**

186 Secondary metabolites of bacterial isolates were extracted with ethyl acetate. A pre-inoculum
187 of the bacteria was performed in 5.0 mL of R2A Broth medium (Himedia ref. 1687) with
188 AWS NaCl 5% and incubated at 28 °C for 5 to 7 days. After the growth of the culture, the
189 total volume was transferred to an Erlenmeyer containing 500.0 mL of the same culture
190 medium and incubated for 7 days under agitation at 150 rpm, followed by 23 days without
191 agitation. After this period, 500.0 mL of ethyl acetate were added to the medium containing
192 the bacterial growth and ruptured in ultra turrax basic (IKA ref. 02H2063.08.CC) at 7,000
193 rpm for 15 min. The organic fraction was obtained and the crude extract was concentrated in
194 the rotary evaporator (R-215 Buchi), under vacuum and temperature of 40 °C, until the
195 solvent was completely dried and stored at 4°C. The crude extracts of three strains
196 representatives were taken for antimicrobial and antiproliferative activity tests.
197 Simultaneously, the crude extracts also were analyzed by liquid chromatography coupled to
198 the mass spectrometer (Versamax, molecular manufacturer Devices).

199

200 **2.4.2 Crude extract fractionation of the extremophilic bacterial.**

201 The crude extracts of the isolates were fractionated using a vacuum chromatographic column
202 with C18. The initial crude extract was solubilized in methanol. The mobile phases used were
203 fraction 1 water (H_2O); fraction 2 water: methanol (H_2O: MeOH 1:1 v/v); fraction 3 methanol
204 (MeOH); fraction 4 MeOH: ethylacetate (EtOAc) (1:1 v/v); fraction 5 EtOAc 100%; fraction
205 6 EtOAc: Glacial acetic acid 1%. These fractions were dried in rotary evaporator at 45 ^{o}C,

206 under vacuum weighted and used for activity antibacterial and antiproliferative tests.

207

208 **2.4.3 *In vitro* antibacterial activity assay**

209 Three crude bacterial extracts (M-92, B-81 and B-146) were tested as antimicrobial producers
210 using the minimum inhibitory concentration (MIC) assay, following the protocol reported by
211 Siddharth and Rai 2018. The crude extracts partially diluted in 1% Dimethylsulfoxide
212 (DMSO) 1 mg/mL – 3.9 µg/mL, and sterile broth were added into pre-coated microbial
213 cultures, completed a total volume of 200 µL. The plate was incubated at 37 °C and room
214 temperature; the lowest concentration of extract, which completely inhibited the bacterial
215 growth was considered as MIC. Each biological assay was performed in triplicate. The
216 pathogenic bacteria used in this test were *Escherichia coli* ATCC 10536, *Staphylococcus*
217 *aureus* ATCC 6538, and *Acinetobacter baumannii* with code AC-972, the strain was not
218 acquired prospectively, the source of origin comes from the bank of MDRs isolated from a
219 patient with pneumonia from the UCI of the Hospital Regional Lambayeque, details such as
220 patient data linked to the sample were anonymized in order to obtain access

221

222 **2.4.4 *In vitro* antiproliferative activity assay**

223 This assay aimed to detect anticancer activities by evaluating antiproliferative action in
224 human tumor cells (Monks et al. 1991). *In vitro* tests with the crude extract of *Streptomyces*
225 sp. M-92, B-81, and B-146 on human tumor cell lines of different origins and a non-tumor
226 cell line. Human tumor cell lines U251 (glioma), UACC-62 (melanoma), MCF-7 (breast),
227 NCI-ADR/RES (ovarian expressing phenotype with multiple drugs resistance), 786-0
228 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), K562

(leukemia) and a non-tumor cell line HaCaT (keratinocyte) were obtained from the National Cancer Institute Frederick, Molecules (MD, USA). Stock cultures were grown in medium containing 5 mL of RPMI 1640 (GIBCO BRL, Gaithers-Burg, MD, USA) supplemented with 5 % fetal bovine serum (FBS, GIBCO) at 37 °C with 5% CO_2. Penicillin: streptomycin (1000 µg·L−1:1000 U·L−1, 1 mL·L−1) was added to the experimental cultures. Cells in 96-well plates (100 µL cells well−1) were exposed to the extracts in DMSO (Sigma-Aldrich) /RPMI (0.25, 2.5, 25, and 250 µg·mL−1) at 37 °C, and 5% CO_2 in the air for 48 h. The final DMSO concentration (0.2% in higher concentration) did not affect cell viability. Before (T_0) and after (T_1) sample application, cells were fixed with 50% trichloroacetic acid (Merck), and cell proliferation was determined by the spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Using the concentration-response curve for each cell line, the values of the sample concentration required to produce total growth inhibition or cytostatic effect (TGI) were determined through non-linear regression analysis using software ORIGIN 8.6® (OriginLab Corporation, Northampton, MA, USA).

2.5 Mass Spectrometry analysis

Streptomyces sp. B-81 extract was resuspended in 1 mL of methanol (HPLC grade) and 100 µL were diluted in 900 µL of methanol. The final solution was filtered through 0.22 µm into vials. Ultra-high pressure liquid chromatography-mass spectrometry (UHPLC-MS) analyses were performed in a Thermo Scientific QExactive® Hybrid Quadrupole-Orbitrap Mass Spectrometer. Parameters: positive mode, capillary voltage at +3.5 kV; capillary temperature at 250 °C; S-lens of 50 V and *m/z* range of 133.40-2000.00. Tandem mass spectrometry (MS/MS) was performed using normalized collision energy (NCE) of 30 eV and 5 precursors

252 per cycle were selected. Stationary phase: Thermo Scientific Accucore C18 2.6 µm (2.1 mm
253 x 100 mm) column. Mobile phase: 0.1% formic acid (A) and acetonitrile (B). Eluent profile
254 (A: B) 0-10 min, gradient from 95:5 up to 2:98; held for 5 min; 15-16.2 min gradient up to
255 95:5; held for 8.8 min. Flow rate: 0.2 mL min^{-1}. The injection volume of the samples was 3
256 µL. Operation and spectra analyses were conducted using Xcalibur software (version 3.0.63)
257 developed by Thermo Fisher Scientific.
258
259 **2.6 Molecular MS/MS network**
260 A molecular network for *Streptomyces sp*. B-81 was created using the online workflow at
261 GNPS (https://gnps.ucsd.edu/). The data were filtered by removing all MS/MS peaks within
262 ± 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top
263 6 peaks in the ± 50 Da window throughout the spectrum. The data was then clustered with
264 MS-Cluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of
265 0.02 Da to create consensus spectra. A network was then created where edges were filtered
266 to have a cosine score above 0.5 and more than 5 matched peaks. Further edges between two
267 nodes were kept in the network only if each of the nodes appeared in each other's respective
268 top 10 most similar nodes. The spectra in the network were then searched against GNPS'
269 spectral libraries. The library spectra were filtered in the same manner as the input data. All
270 matches kept between network spectra and library spectra were required to have a score
271 above 0.5 and at least 5 matched peaks (Wang et al. 2016).
272

273 3. Results and discussion

3.1 Isolation, identification and selective of bacterial species from the northern saline lagoons of Perú

In total, 166 pure cultures showing different colony morphologies were obtained grown on R2A medium from saline water (A), water and sediment (AES), and sediment (SE). The morphological characteristics such as aerial mycelium, the morphological spore mass color, pigmentation of vegetative or substrate mycelium, and the production of diffusible pigment were used to classify 42 filamentous and 8 non filamentous to the genera *Streptomyces* 39 (78%), *Pseudonocardia* 3 (6%), *Staphylococcus* 4 (8%), *Bacillus* 2 (4%), and *Pseudomonas* 2 (4%) in Table 2, (Flores, 2017).

Table 2. 16S rRNA gene-based identification of bacteria isolate from lagoons Morrope and Bayovar.

Sample type	Number of isolate (%)	Phylum	Family	Genus
AES, SE	39 (78%)	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
SE	3 (6%)	*Actinobacteria*	*Pseudonocardiaceae*	*Pseudonocardia*
A	2 (4%)	*Firmicutes*	*Bacillaceae*	*Bacillus*
A	4 (8%)	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus*
AES	2 (4%)	*Proteobacteria*	*Pseudomonadaceae*	*Pseudomonas*

The isolates were regrouped into 28 isolates due to their similar phenotypic characteristics and were identified based on the sequencing and alignment, from the 16S rRNA gene analyzes. They belong to the genus *Streptomyces* 27 isolates and 1 *Pseudonocardia* isolate, and 23 isolates showed a 16S rRNA gene sequence similarity below the 98.7% threshold for proposing as novel species (Table 3).

Table 3. Strain Actinobacterial isolate from different sample Bayovar and Mórrope saline lagoons, indicating the top hit taxon, sequences length nucleotides, and similarity percent of the isolates the "Identify" service best-hit classifier in the EZBioCloud 16S Database.

	EZBioCloud Hit Strain name	Top Hit taxon	16S rRNA sequences length (nt)	Similarity %
Mórrope Zona 1				
M-92	TRM 45540	*S. luteus*	1313	99.16
M-142	NBRC 15864	*S. labedae*	1440	97.58
Mórrope Zona 2				
M-239	TRM 45540	*S. luteus*	1472	99.19
M-239.2	TRM 45540	*S. luteus*	1508	99.19
M-261	LMG 19316	*S. griseoincarnatus*	1468	97.58
M-262	NBRC 12780	*S. griseorubens*	1440	97.57
M-266	NBRC 12825	*S. variabilis*	1345	97.58
M-270	NBRC 13433	*S. pactum*	1471	97.98
M-274.2	LMG 19316	*S. griseoincarnatus*	1471	97.58
M-275	NRRL B-3009	*S. olivaceus*	1380	97.98
M-282.1	NRRL B-3009	*S. olivaceus*	1412	97.98
M-285	NRRL B-3009	*S. olivaceus*	1507	97.98
M-289.1	NBRC 13433	*S. pactum*	1441	97.98
M-290	OU-40	*S. hyderabadensis*	1481	98.06
M-290.1	NRRL B-3009	*S. olivaceus*	1398	97.98
Mórrope Zona 3				
M-323	NRRL B-3009	*S. olivaceus*	1500	97.98
Bayovar Zona 4				
B-81	NRRL B-3009	*S. olivaceus*	1345	99.93

B-93.1	ISP 5183	*S. violascens*	1339	97.37
B-93.3	LMG 19316	*S. griseoincarnatus*	1498	97.58
B-95	LMG 19316	*S. griseoincarnatus*	1456	97.58
B-95.1	NBRC 15864	*S. labedae*	1359	97.58
B-236.1	YIM 63235	*Pseudonocardia antimicrobica*	1500	98.41
B-144.2	NRRL B-3009	*S. olivaceus*	1519	97.98
B-146	NRRL B-3009	*S. olivaceus*	1287	99.84
B-236	LMG 19316	*S. griseoincarnatus*	1516	97.58
B-244	NRRL B-3009	*S. olivaceus*	1463	97.98
B-265	LMG 19316	*S. griseoincarnatus*	1391	97.58
B-379	NBRC 13475	*S. hydrogenans*	1493	97.27

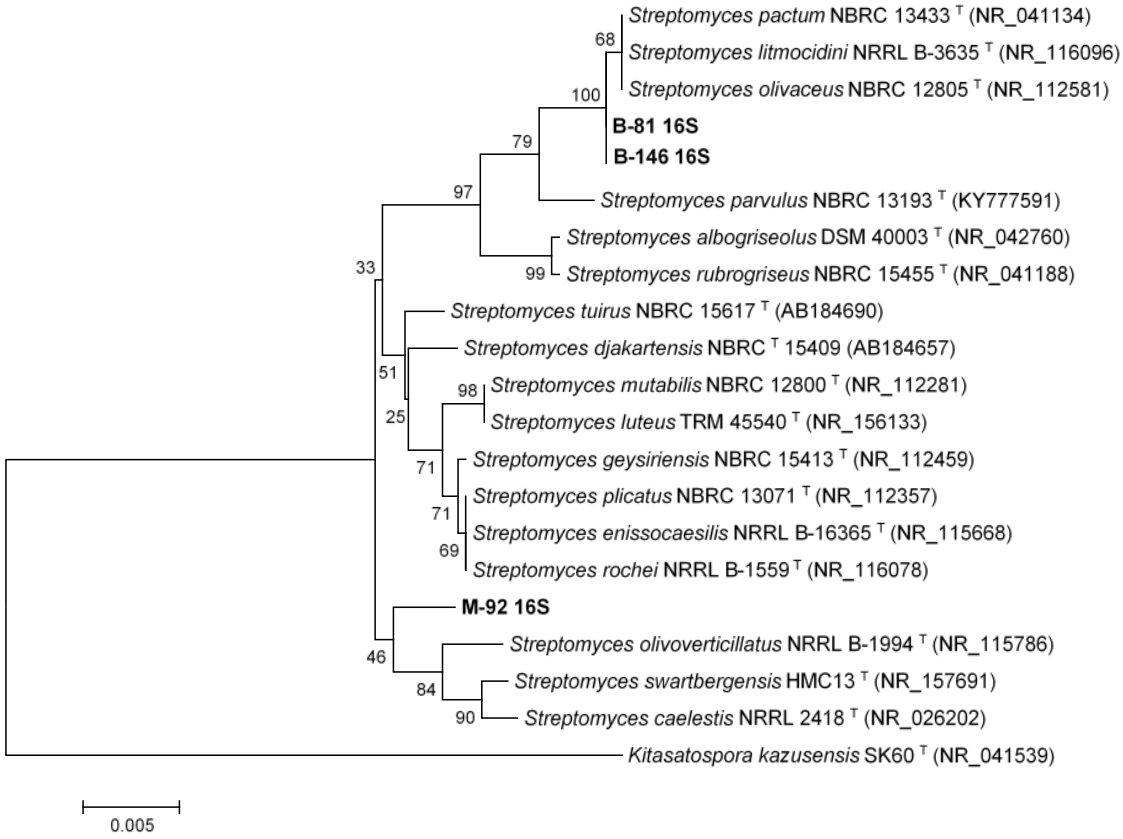
297

298 Therefore, these strains should be designed for further taxonomic and analytical chemistry
299 analyses to confirm their novelty at species rank and as a source of novel chemical entities.
300 Two isolates were identified in lagoon 1 of the Morrope salt flats, and according to the
301 EZBioCloud the isolate M-92 presented similarity with *Streptomyces luteus* TRM 45540
302 (99.16%), while the isolate M-142 presented similarity with *Streptomyces labedae* NBRC
303 15864 (97.58%). In lagoon 2 of the Morrope salt flats, 13 isolates were identified, of which
304 M-239 and M-239.2 were similar to *S. luteus* TRM 45540 (99.19%), the isolates M-261 and
305 M-274.2 presented similar to *S. griseoincarnatus* LMG 19316 (97.58%), the isolate M-262
306 showed similarity to *S. griseorubens* NBRC 12780 (97.57%), the isolate M-266 was similar
307 to *S. variabilis* NBRC 12825 (97.58%), the isolates M-270 and M- 289.1 were similar to *S.*
308 *pactum* NBRC 13433 (97.98%), the isolate M-290 presented similar to *S. hyderabadensis*
309 OU-40 (98.06%), and finally four isolates, M-275, M-282.2, M-285 and M- 290.1 were
310 similar to *S. olivaceus* NRRL B-3009 (97.98%). From the lagoon 3, also belongs to the
311 Morrope salt flats, it was identified one isolate M-323 similar to *S. olivaceus* NRRL B-3009
312 (97.98 %). Finally, from lagoon 4, belongs to the Bayovar salt flats, 12 isolates were

313 identified, four isolates were similar to *S. olivaceus* NRRL B-3009, B-81 (99.93%0, B-144.2
314 (97.98%), B-146 (99.84%), and B-244 (97.98%). Also, in the lagoon 4 the isolate B-93, was
315 similar to *S. violascens* ISP 5183 (97.37%), and other four isolates presented similar *S.*
316 *griseoincarnatus* LMG 19316, B-93.3, B-95, B-236, B-265 (97.58%), the isolate B-95.1 was
317 similar to *S. labedae* NBRC 15864 (97.58%), and the isolate B-379 was similar to *S.*
318 *hydrogenans* NBRC 13475 (97.27%). Besides Streptomyces, another of the actinobacteria
319 genus was recovered to the Bayovar salt flats. The isolate B-236.1 was similar to
320 *Pseudonocardia antimicrobica* YIM 63235 (97.27%) and our work is the first to report this
321 genus present in the Bayovar salt flats (Table 3).
322 In the Morrope salt flats, eight different groups of *Streptomyces* were identified, whereas in
323 the Bayovar saline only five groups of *Streptomyces* were recovered, however, a different
324 genus like *Pseudonocardia*, whichhas been reported by Zhang et al. (2016) as producers of
325 γ-Butyrolactones that previously had only been reported for Streptomyces genera and in
326 plants was recovered in this study (Table 3). Our culture-based approach using a pre-
327 enrichment to decrease the bacterial load agrees with the reports described in the literature,
328 in which the isolation of halophilic bacteria reveals low species richness and the dominance
329 of *Streptomyces* genus Parthasarathi et al. (2012). Ballav et al. (2015) reported *Streptomyces*
330 spp. as the most predominant group contributing to 46% of the total isolates in crystallizer
331 pond sediments of Ribandar saltern, Goa, India. In this study the isolation was performed
332 with three different types of culture media and four different concentrations of salt because
333 in lagoons were found the concentration of salts between 0-300 psu units (Ballav et al. 2015).
334 Cortes et al. (2019) in the Salar de Huasco report similar results, they were isolated
335 *Streptomyces* (86%), *Nocardiopsis*, (9%), *Micromonospora* (3%), *Bacillu*s (1%) and

336 *Pseudomonas* (1%).

337 The phylogenetic analysis based on their nearly complete 16S rRNA gene sequences (>1206
338 bp) was constructed with the isolates M-92, B-81 and B-146 because them presented
339 antibacterial and antiproliferative activities. The phylogenetic tree showed clades well
340 supported to the three isolates and the reference sequences (Figure 3).



341

342 **Fig 3. Phylogenetic tree based on 16S rRNA gene sequences (1206 positions in the final**
343 **dataset) inferred using the neighbour-joining method in MEGA7, the evolutionary**
344 **distances were computed using the Kimura 2-parameter method, showing the**
345 **phylogenetic positions of different saline isolates and type strains within the genus**
346 **Streptomyces and Pseudonocardia. Numbers at branching points refer to percentages**

of bootstrap values from 1000 replicates. The scale bar indicates 0.02 indicated substitutions per nucleotide. *Kitasospora kazusensis* SK60^T (NR_041539) was used as outgroup.

Isolate M-92 forms a distinct branch closely related to the type strain of *Streptomyces olivoverticulatus* NRRL-1994^T, *Streptomyces swartbergensis* HMC13^T, and *Streptomyces caelestis* NRRL-2418^T. Isolates B-81 and B-146 form a well-supported sub-clade closely associated to *Streptomyces olivaceus* NBRC 12805^T, *Streptomyces litmocidini* NRRL B-3635^T, and *Streptomyces pactum* NBRC 13433^T (Figure 3). The length of the branch of all *Streptomyces* isolates in the phylogenetic tree and the assignation to these isolates to completely diff erent clades from each other, highlight the divergence of them from their closely related neighbors. Further studies need to be performed to confirm the right affiliations of these isolates to the novel species within the evolutionary radiation of the genus *Streptomyces*.

3.2 Antibacterial activity Screening

Minimum Inhibitory Concentration (MIC) assay was used to screen antibacterial metabolites producer strains against the indicator strains *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538, and *Acinetobacter baumannii* AC-972 MDR; the crude extracts tested from the three extremophilic bacterial reported positive results against the pathogenic bacteria tested. The antimicrobial activity of the crude extract of *Streptomyces* sp. B-81 strain present the highest inhibitory activity with the lowest MIC with 7.82 µg/mL against the three pathogens, followed by *Streptomyces* sp. M-92 showed MIC of 7.82 µg/mL against *E. coli* ATCC 10536 and *S. aureus ATCC 6538*, and 15.63 µg/mL against *A. baumannii AC-972*

370 MDR. *Streptomyces* sp. B-146 showed MIC of 15.63 µg/mL against the pathogens tested. It
371 is important to highlight that all crude extracts of the strains presented low MIC in
372 comparison with the reference antibiotic (Table 4).
373
374 **Table 4. Minimum inhibitory concentration MIC antibacterial of three *Streptomyces* sp.**
375 **crude extracts, by broth dilution method.**

Isolates	**Minimum Inhibition Concentration(ug/mL)**			
	E. coli ATCC 10536	*S. aureus ATCC 6538*	*A. baumannii AC-972* MDR	Antibiotic Chloramphenicol
M-92	7.82	7.82	15.63	22,2
B-146	15.63	15.63	15.63	23,1
B-81	7.82	7.82	7.82	22,3

376
377 Similar results were reported in a marine *Streptomyces* sp. S2A with MIC 31.25 µg/mL
378 against *Klebsiella pneumoniae*, 15.62 µg/mL against *Staphylococcus epidermidis*,
379 *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli* and *Micrococcus luteus* with 7.8
380 µg/mL (Saket Siddharth and Ravishankar R. Vittal, 2018). The crude extract of the
381 *Streptomyces* sp. YBQ59 strain against nine pathogens, obtaining MIC results between 10.5
382 to 22.5 µg/mL, their results being similar to those we obtained (Vu. H et al. 2018).
383
384 **3.3 Antiproliferative activity Screening**
385 The antiproliferative activity of the crude extracts of *Streptomyces* sp. M-92, B-81, and B-
386 146 against three human tumor cell lines were expressed as TGI (Total Growth Inhibition-
387 concentration that inhibited cell growth by 100%) (Table 5).
388

Table 5. Antiproliferative activity of three Streptomyces extracts against human tumor cell lines.

Crude extract and positive control	TGI (µg/mL)[c]		
	U251	**MCF-7**	**NCI-H460**
Doxorubicin[a]	2.46	6.11	<0,025
M-92	5.06	7.96	9.33
B-146	5.41	8.03	5.06
Doxorubicin[b]	1.50	>25	1.80
B-81	0.57	0.80	0.61

Human tumor cell lines: U251 (glioma), MCF-7 (breast), NCI-H460 (lung of type non-small cells)
[a, b] Doxorubicin used as positive control;
[c] TGI Concentration that totally inhibited cell growth

According to Abreu et al. (2014), TGI values higher than 50 µg/mL represent inactive samples. In comparison to the others *Streptomyces* tested and doxorrubicin, *Streptomyces* sp. B-81 shown the best result against glioma (TGI =0,57 µg/mL), lung cancer (TGI =0,61 µg/mL) and breast cancer (TGI =0,80 µg/mL). Due to the result obtained with the crude extract of *Streptomyces* sp. B-81 strain, the extracts from three different media were selected for further fractionation, to have access to the metabolic profile. It was possible to verify that the highest activity detected was in the extracts from the fermentation of *Streptomyces* sp. B-81 in the R2A medium (Table 5), which inhibited the nine different types of tumor cell lines. However, it is observed that the fractionation decreases the antitumor activity presented in the crude extract and the putative active compounds are recovered in fractions 3 and 4. Fraction 3 seemed to contain antiproliferative substances in lower concentration and was able to inhibit U251 (glioma) (TGI = 38.3 µg/mL), OVCAR-03 (ovary) (TGI = 62.1 µg/mL), and K562 (leukemia) (TGI = 81.5 µg/mL), and fraction 4 inhibited U251 (glioma) (TGI = 73.5 µg/mL) and UACC-62 (melanoma) (TGI = 89,4 µg/mL) (Table 6).

Table 6. Antiproliferative evaluation of isolate *Streptomyces* sp. B-81 fermented in three different medium and fractions of the R2A medium against nine tumors and one non-tumor cell line.

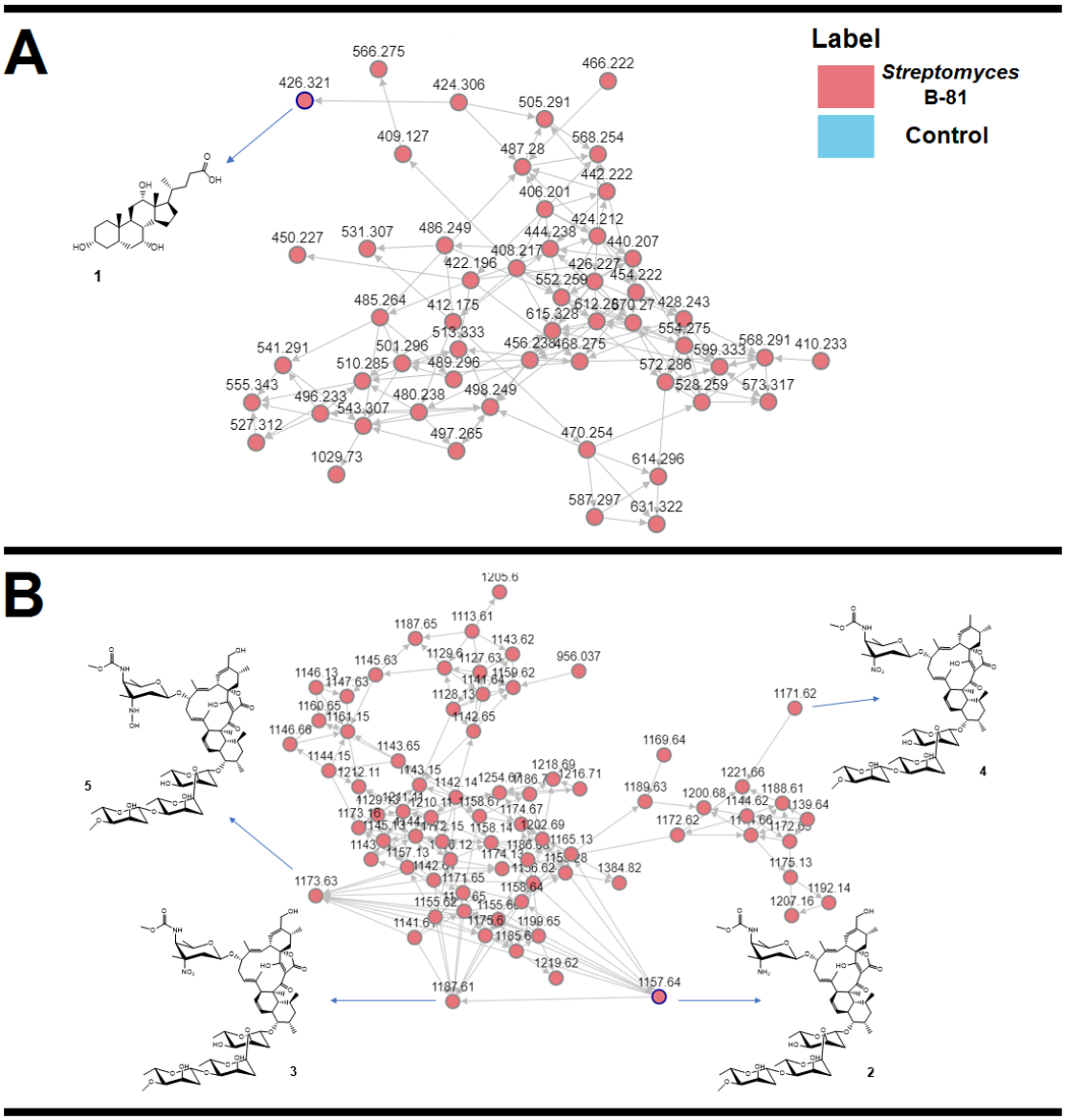
	TGI (µg/mL)									
Crude extract	**2**	**u**	**m**	**a**	**7**	**4**	**o**	**h**	**k**	**q**
Doxorubicin	0,03	0,76	>25	>25	0,22	>25	14,4	>25	6,3	>25
B-81 NB	70,9	250	*	*	*	*	*	*	*	*
B-81 ISP2	14,1	17,2	*	155,9	*	38,9	55,2	155,7	*	*
B-81 R2A	1,6	21,3	8,0	31,0	39,0	11,2	19,4	89,7	11,6	158,1
B-81 R2A fractions	**2**	**u**	**m**	**a**	**7**	**4**	**o**	**h**	**k**	**q**
Fraction 1	*	*	*	*	*	*	*	*	*	*
Fraction 2	*	*	*	*	*	*	*	*	*	*
Fraction 3	38,3	127,2	154,6	189,8	172,6	108,0	62,1	167,4	81,5	*
Fraction 4	73,5	89,4	184,5	143,2	*	235,1	81,6	195,2	100,4	*
Fraction 5	*	*	*	*	*	*	*	*	*	*
Fraction 6	163,0	*	*	*	*	*	*	*	*	*

B-81 NB: *Streptomyces sp.* Crude extract in nutrient broth growth medium. B-81 ISP2: *Streptomyces sp*. Crude extract in yeast extract-malt. B-81 R2A: *Streptomyces sp*. Crude extract in R2A growth medium. B-81 R2A fractions: 1 = H_20; 2 = MeOH-H_2O; 3 = MeOH; 4 = MeOH-EtOAc; 5 = 100% EtOAc; 6 = EtOAc -acid: Human tumor lines: 2 = U251 (glioma), u = UACC-62 (melanoma); m = MCF-7 (breast); a = NCI-ADR / RES (ovary with multiple drug resistance phenotype); 7 = 786-O (kidney adenocarcinoma); 4 = NCI-H460 (lung, non-small cell type); o = OVCAR-03 (ovary); h = HT-29 (colon); k = K562 (leukemia). Human non-tumor lineage: q = HaCaT (keratinocyte).
Doxorubicin (Reference chemotherapy).
TGI: Total Growth Inhibition- Concentration necessary to completely inhibit cell proliferation.
* TGI >250 µg/mL

It is common to find this antiproliferative activity in the *Streptomyces* genus due to its metabolic and genetic capacity to produce secondary metabolites (Tan and Liu, 2017). Additionally, actinomycetes isolated from marine environments have a greater capacity to express these metabolites in response to the pressure of environmental selection to which

427 they have been subjected throughout the evolutionary history of aquatic organisms (Lam,
428 2006). This pressure has generated high specificity and a complex three-dimensional
429 conformation of the compounds to act in the marine environment (Song et al, 2018). In this
430 particular case, the geographical isolation, the geological conditions of formation of the
431 saline lagoon and the extreme environmental conditions generate a greater selection
432 environmental pressure, which makes it possible to observe antibiotic and antiproliferative
433 activity in the isolates analyzed.
434 The anticancer potential of halophilic actinomycetes has been extensively studied. Secondary
435 metabolites Salternamides A-D isolated from halophilic actinomycetes of saline on the island
436 of Shinui (Republic of Korea), have potential cytotoxicity against the human colon cancer
437 cell line (HCT116) and gastric cancer cell line (SNU638) (Kim et al. 2015). Another
438 moderately halophilic *Streptomyces sp*. W1 with number accession GenBank JN187420.1,
439 isolated from Weihai Solar Saltern in China, has potent cytotoxicity against human lung
440 cancer cell line (A549) with IC_{50} 78.6 µM; human cervical epithelial cell line (HeLa) IC_{50}
441 56.6 µM, human liver cancer cell line (BEL-7402) with IC_{50} 47.1 µM, and human colon
442 cancer cell line (HT-29) with IC_{50} 94.3 µM (Liu et al. 2013). Four compounds called
443 shellmicin A-D isolated from the *Streptomyces* sp. shell-016 were described by (Yong Han
444 et al. 2020) and studied concerning cytotoxicity against five human tumor cells lines: non-
445 small cell lung cancer (H1299, ATCC CRL-5803), malignant melanoma (A375, ATCC CRL-
446 1619), hepatocellular carcinoma (HepG2, ATCC HB-8065), colorectal adenocarcinoma
447 (HT29 ATCC HTB-38) and breast cancer (HCC1937, ATCC CRL-2336). The study
448 indicated that the compounds Shellmicin A, B and D showed greater cytotoxicity than
449 Shellmicin C compound, with an IC_{50} ranging from 0.69 µM to 3.11 µM at 72 h. An

450 interesting fact was that shellmicin C and D are a pair of stereoisomers and their biological
451 activity was significantly different. It is worth mentioning that in our study we determined
452 that the activity of the crude extract was better than that of the fractions, the same ones that
453 presented a different activity, therefore, we can deduce that we would be facing compounds
454 with synergism.
455 An actinomycete strain designated as *Streptomyces* sp. KML-2 isolated from a saline soil
456 mine in Khewra, Pakistan, showed antitumor activity against Hela cells (cervical cancer) and
457 MCF-7 (breast cancer) cell lines with IC_{50} values of 8.9 and 7.8 µg/mL respectively in the
458 initial screening, then they carried out a higher production (20 L) for the purification of the
459 compounds, confirming a wonderful anti-tumor potential against MCF-7 cells with an IC_{50}
460 value of only 0.97 µg /mL (Usman et al. 2015). This study also revealed that the Khewra salt
461 mine from which the KML-2 strain was isolated is a powerful ecological niche with
462 inimitable strain diversity that has yet to be discovered, a fact that reinforces the approach of
463 our study in the search for new compounds with biotechnological activity.
464
465 **3.4 Secondary Metabolite Analysis of *Streptomyces sp*. B-81 isolate.**
466 The crude extract obtained from *Streptomyces* sp. B-81 was investigated through UHPLC-
467 MS/MS analyses and demonstrated a broad profile of biomolecules (Fig. S1A). Furthermore,
468 a metabolite screening in the GNPS platform was performed and molecular networking
469 revealed two clusters (A and B) that exhibited secondary metabolites produced by
470 *Streptomyces sp*. B-81 extract (pink) (Fig. 4).



471

472 **Fig 4. Molecular networking obtained for *Streptomyces* sp. B-81 extract. Cholic acid and**

473 **derivatives were grouped in cluster A, and lobophorins were grouped in cluster B.**

474 **Nodes circled in blue indicate molecules identified by comparison with the GNPS**

475 **platform database.**

476

Metabolites were identified as a hit in the GNPS database or manually identified by accurate mass analyses, which showed mass errors below 5 ppm (Table 7).

Table 7. MS data obtained for secondary metabolites detected in *Streptomyces* sp. B-81 extract.

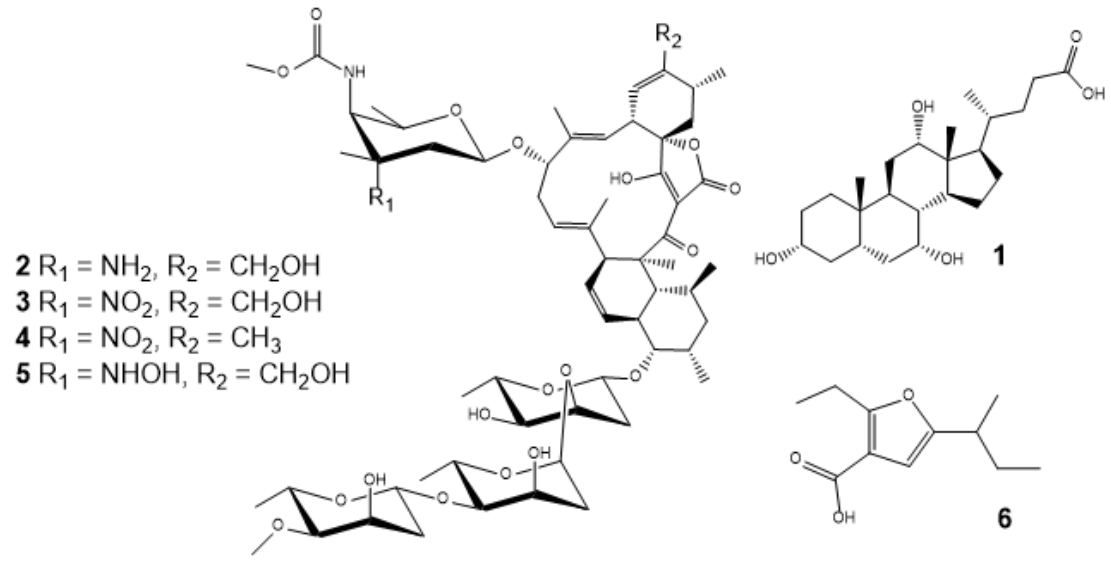
Compound	Ion formula	Calculated *m/z*	Experimental *m/z*	Error (ppm)
Cholic acid	$C_{24}H_{44}NO_5$	426.3219	426.3212	-1.6
Lobophorin A	$C_{61}H_{93}N_2O_{19}$	1157.6372	1157.6373	0.1
Lobophorin B	$C_{61}H_{91}N_2O_{21}$	1187.6114	1187.6108	-0.5
Lobophorin E	$C_{61}H_{91}N_2O_{20}$	1171.6165	1171.6160	-0.4
Lobophorin K	$C_{61}H_{93}N_2O_{20}$	1173.6322	1173.6321	-0.1
Compound 6	$C_{11}H_{17}O_3$	197.1177	197.1173	-2.0

The observed signals corresponded to cholic acid (1), lobophorin A (2), lobophorin B (3), lobophorin E (4) and lobophorin K (5).

In cluster A, GNPS database indicated the presence of cholic acid in the *Streptomyces* sp. B-81 extract (Fig. S2 and S3). In the molecular networking, each MS/MS spectra are represented by nodes, which are grouped in clusters based on their fragmentation pattern similarity. Thus, compounds of the same class of molecules are grouped in the same cluster (Wang et al. 2016). As observed in Figure 4, the cholic acid cluster is composed by a range of nodes, suggesting the presence of other analogues. Cholic acid and other bile acids have been reported as secondary metabolites of specific members of the genus *Streptomyces* and *Myroides* (Kim et al. 2007). In the literature, cholic acid-derivatives and bile acids are known as antimicrobials agents, displaying activity against gram-negative and positive bacteria or

494 improving the antimicrobial effect of antibiotics (Schimidt et al. 2001, Rasras et al 2010,
495 Darkoh et al. 2010, Savage et al. 2000).
496 In cluster B, GNPS database indicated the production of lobophorin A by *Streptomyces* sp.
497 B-81 strain (Fig. S4 and S5). Also, MS/MS profile of compound **2** presented *m/z* 883.49 and
498 753.43 (Fig. S5), related to the loss of the sugar units in the structure, according with the
499 literature (Nguyen et al. 2020). Similar as observed in cluster A, cluster B also exhibited
500 other lobophorin compounds such as lobophorins B, E and K that were identified based on
501 their accurate masses and fragmentation profiles with typical fragments at *m/z* 184.09,
502 157.09, 108.08 and 97.06 (Fig. S6-S8). Lobophorins were discovered by Jiang et. al. (1999)
503 in the expedition to Belize on board of the Columbus research ship. In the occasion, a new
504 strain of actinomycete, named as # CNC-837, was isolated from the surface of a brown algae
505 from the Caribbean called *Lobophora variegata*, and was reported to produce lobophorins A
506 and B, potent anti-inflammatory agents (Jiang et al. 1999). Besides lobophorins A and B,
507 other compounds of this class have been reported in the literature. Lobophorins C and D have
508 similar structures to A and B, while Lobophorin C exhibited potent cytotoxic activity against
509 human liver cancer cells, lobophorin D displayed significant inhibitory effect on human
510 breast cancer cells (Wei et al. 2011). Lobophorins E and F were reported by Niu et al. 2011,
511 that also described antibacterial and cytotoxic activities for lobophorin F. In other study, Pan
512 et al. 2013 reported lobophorins H and I and described that lobophorin H exhibited
513 antibacterial activities against *Bacillus subtilis*. Most recent, Lobophorin K was reported
514 (Braña et al. 2017) and also displays antimicrobial and cytotoxic activities.

515 Besides cholic acid and lobophorins, a furan-type compound (6) were also detected in the
516 *Streptomyces sp*. B-81 extract (Fig. S9). The structures of the metabolites **1-6** are shown in
517 Fig. 5.



518

519 **Fig 5. Structures of secondary metabolites identified in *Streptomyces* sp. B-81 extract.**

520

521 Compound 6 exhibited fragments at m/z 168.07, 167.07 and 153.05, in agreement with the
522 literature (Nguyen et al. 2020). Compound 6 was reported as a secondary metabolite of the
523 *Streptomyces sp*. VN1 strain, a microorganism isolated of sea sediment from a coastal region
524 of Viet Nam (Nguyen et al. 2020). In addition, *Streptomyces sp*. VN1 was also described to
525 produce lobophorin A (Nguyen et al. 2020). Nguyen et al 2020, reported that compound 6
526 displayed cytotoxic activity, *in vitro*, against 5 types of tumor cell lines. Furthermore, Wang
527 et al. 2008 reported that other furan compound named HS071, produced by *Streptomyces sp*.
528 HS-HY-071, displayed anticancer activity.

529

Our data deserves further investigation in order to correlate the antimicrobial and cytotoxic activities, observed for the *Streptomyces* sp. B-81 extract, to the bile acids, lobophorins and furan compounds. In addition, the extremophilic environments, in this particular case the Salinas de Bayovar, are prolific sources of microorganisms, such as unique *Streptomyces*. Due the extreme conditions, these microorganisms produce different compounds in order to adapt to survive in the environment, which makes *Streptomyces* sp. B-81 an attractive source of bioactive compounds.

Conclusion

The present study was successful in determining the diversity and bioactive potential of the actinobacterial isolates present in this type of environment (Mórrope and Bayovar salt flats) located in northern Peru, which are environments that had not been explored for this type of study. this being the first study. Furthermore, it has been found that the isolates of actinobacetria *Streptomyces sp*. M-92, B-146 and B-81 may be new species, their importance for these isolates lies in their antibacterial and antiproliferative potential, being the most promising for their best activity *Streptomyces sp*. B-81. Six biomolecules (colic acid, Lobophorin A, B, E and K in addition to a sixth compound) were detected in this cultivable actinobacterial isolated from Bayovar's salt flats, but not yet named from the cured base GNPS. With this study it is determined that the actinobacteria that live in these environmental conditions present an enormous wealth of microorganisms that are new species that produce new and biologically active compounds and contribute to the study of extreme environments such as the saline lagoons of northern Peru with antibacterial potential. and antiproliferative.

552 Further studies will be carried out in the identification of these molecules in order to purify
553 and characterize them since this can result in the economic production of bioactive
554 compounds for future pharmaceutical applications.

555

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567

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