1   **EVALUATION OF ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES**

2   **OF ACTINOBACTERIA ISOLATED FROM THE SALINE LAGOONS OF**

3   **NORTHWEST PERU.**

4

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28

Abstract

30   The unexplored saline lagoons of the north of Peru harbor a rich microbiome, due to reported

31   studies of different extreme environments around the world. In these regions, there are

32   several ecosystems and microhabitats not yet explored, and little is known about the diversity

33   of actinobacteria and other microorganisms. We suggest that the endemic bacteria present in

34   this extreme environment could be source of active molecules with anticancer, antimicrobial,

35   antiparasitic properties. Using phenotypic and genotypic characterization techniques

36   including the 16S rRNA were identified into the genera *Streptomyces* 39 (78%),

37   *Pseudonocardia* 3 (6%), *Staphylococcus* 4 (8%), *Bacillus* 2 (4%), and *Pseudomonas* 2 (4%).

38   All isolated bacteria for the genotypic data were preliminarily identified. Actinobacteria

39   strains were found dominantly in both sites (Lagoon1-3 = 16 isolates and lagoon 4 = 12

40   isolates). Phylogenetic analysis revealed that 28 isolates were exclusively affiliated to eleven

41   different clusters of Actinobacteria of the major genus *Streptomyces*. Three *Streptomyces* sp.

42   strains M-92, B-146, and B-81, were tested for antibacterial and antiproliferative activities.

43   The results showed antiproliferative activities against three tumor cell lines, U251 glioma;

44   MCF7 breast; NCI-H460 lung non-small type of cells, and the antibacterial activity to

45   *Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 10536, and *Acinetobacter baumanni* AC-

46   972 which is resistant to multiple drugs. The promising results belong to *Streptomyces* sp. B-

2

47   81 strain in the R2A medium using a doxorubicin with control positive, the best result was

48   from the latter (TGI = 0,57 µg/mL) for glioma; NCI-H460 lung of type non-small cells (TGI

49   = 0,61 µg/mL), and breast cancer (TGI =0,80 µg/mL), this strain was selected to be

50   fractionated because it had better antiproliferative and antibacterial activity, and its fractions

51   were evaluated concerning antiproliferative activity against nine types of tumor cells and one

52   non-tumor. The methanolic fraction showed a better result in the antiproliferative activity

53   and was able to inhibit U251 (glioma) (TGI = 38.3 µg/mL), OVCAR-03 (ovary) (TGI = 62.1

54   µg/mL), and K562 (leukemia) (TGI = 81.5 µg/mL). The methanol 50% - acetate 50% fraction

55   (Fraction 4) inhibited U251 (glioma) (TGI = 73.5 µg/mL) and UACC-62 (melanoma) (TGI

56   = 89.4 µg/mL). Moreover, the UHPLC-MS/MS data and molecular networking of

57   *Streptomyces sp*. B-81 isolate extract revealed the production cholic acid, Lobophorin A,

58   Lobophorin B, Lobophorin E, Lobophorin K and compound 6. Extremophilic environments

59   such as the Mórrope and Bayovar Salt Flats are promising sources of new bacteria with

60   promising pharmaceutical potential; These compounds could be useful to treat various

61   infectious diseases or even some type of cancer.

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

63   antimicrobial activity; antiproliferative activity.

64   1. Introduction

65   Perú has extreme environments such as the salt marshes located on the coast, center, and

66   south of the country. In these regions, there are several ecosystems and microhabitats, whose

67   studies are still restricted and report the diversity of bacteria and other organisms (Caceres

68   and Legendre, 2009; Flores and Pisfil, 2014; Leon et al. 2016). Among other

69 microorganisms, the members of the phylum Actinobacteria can be found in all kinds of

70 extreme environments. *Micromonospora*, *Actinomadura*, and *Nocardiopsis* were reported

71 from saline soils of the ephemeral salty lakes in Buryatiya (Lubsanova et al. 2014).

72 *Streptomyces*, *Nocardiopsis*, and *Nocardioides* were isolated from the Regions Western

73 Ghats of India (Siddhart et al. 2020). Concurrent to this, there is still little information

74 documented on isolated substances and their biological activities of the microorganisms

75 isolated from saline environments. *Micromonospora*, *Streptomyces*, *Salinispora*, and *Dietzia*

76 were isolated from the coastal zone of the geographically remote young volcanic Easter

77 Island Chile. Besides, actinobacterial halophilic and halotolerant strains show heterogeneous

78 physiological characteristics among different genera because these bacteria have the ability

79 to synthesize secondary metabolites to cope with conditions of high salinity and the different

80 temperatures present in their environments (Binayke et al. 2018; Oueriaghli et al. 2018;

81 Boyadzhieva et al. 2018). These extreme conditions favor the development of metabolically

82 competitiveness to the production enzymes, which can help the bacterial population to adapt

83 to high salinity (Kim et al. 2019). Furthermore, these microorganisms can also perform

84 essential processes such as carbon cycle, metal transfer, and the removal of organic pollutants

85 to higher trophic levels (Fiedler et al. 2005; Blunt et al. 2007). In addition, this microbial

86 group presents a unique ability to produce new products, mainly antibiotics (Berdy, 2005;

87 Strohl, 2004).

88 The search for these microorganisms has been mainly related to the production of antibiotics

89 and antitumor substances (Newman and Cragg, 2007). There are about 22.500 biologically

90 active substances obtained from microorganisms, 45% of which are represented by

91 actinomycetes, with *Streptomyces* being the producer of 70% of them (Hayakawa et al. 2007

92   and Valliappan et al. 2014). According to Lam (2006), there are new actinobacteria from

93   habitats not yet explored or little explored to be the source of new bioactive secondary

94   metabolites.

95   The bioguided study strategy helps us find a wide variety of microorganisms that have

96   biotechnological potential (Maldonado et al. 2005). This study reports the isolation and

97   phylogenetic analysis of a collection of bacterial isolates from the saline lagoons from the

98   Northern Peru, and their potential as producers of secondary metabolites with antimicrobial

99   and antiproliferative activities.

100

101   2. Materials and methods

102   2.1 Site description and sampling

103   Sample collection at Mórrope saline lagoons was carried out in December 2012 the zone "1",

104   in January 2013 the zone "2", and in July 2014 the zone "3", while samples were collected at

105   Bayovar saline lagoons in March 2015 the zone "4" (Figure 1). The present work did not

106   need permits from a competent authority, since it does not include any protected species,

107   because the standard is currently in the process of being implemented to achieve its

108   registration, likewise the sampling areas are lagoons that due to their nature are in the process

109   of adjudication to the competent governmental organism through the Peruvian Ministry of

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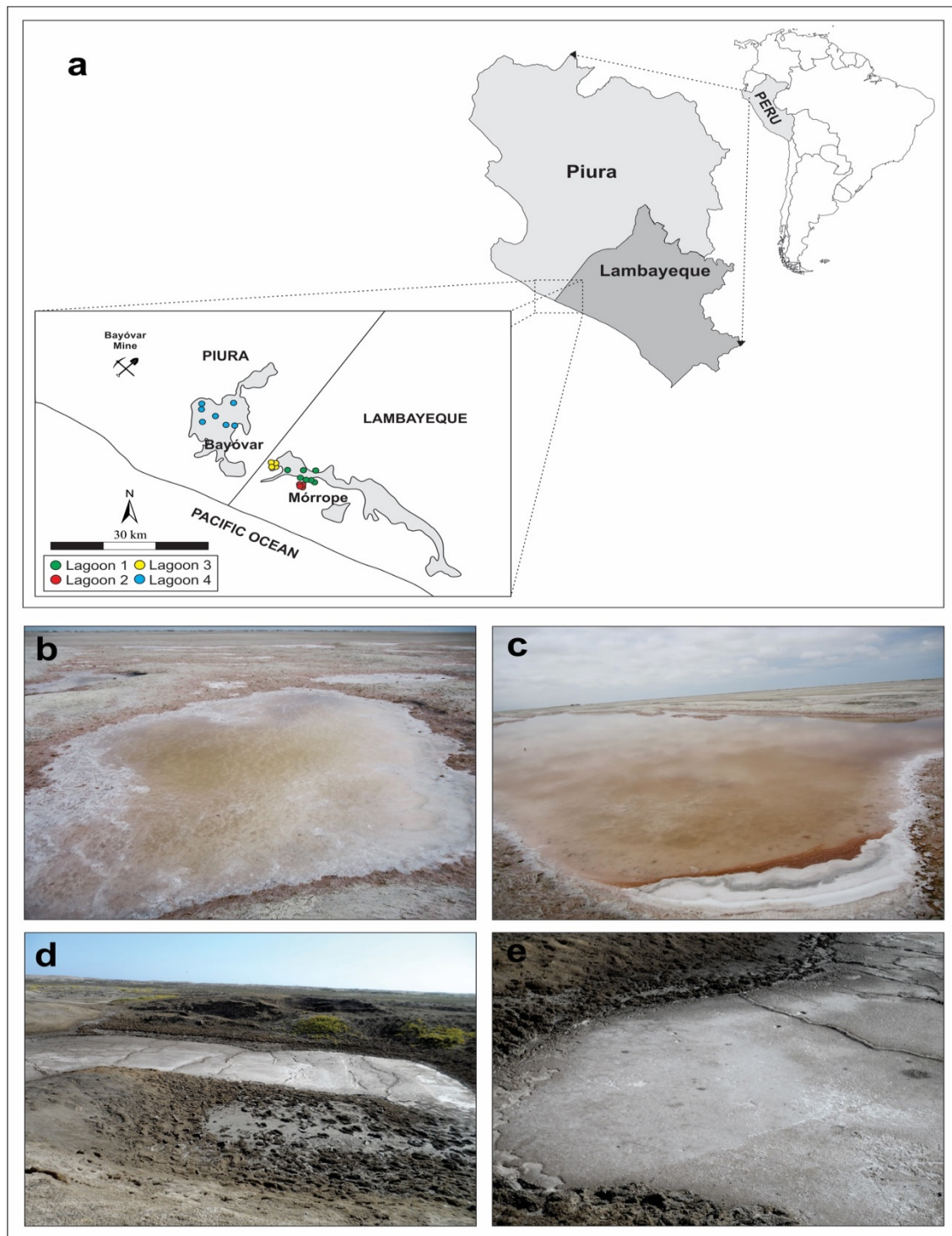
Fig 1. a = Geographic location of sampling sites; b and c = lagoons 1, 2 and 3 (State Mórrope); and e = lagoon 4 (State Bayovar).

115   The sources and places from which samples were obtained are detailed in Table 1. Samples

116   were collected aseptically, placed in sterile plastic bags, and kept refrigerated at 4 °C for

117   further processing in the Laboratory of Bacteriology the Regional Hospital of Lambayeque,

118   a flowchart depicting the methodological strategy adopted in this work is shown in Figure 2.

119

120   **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)

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

122

123
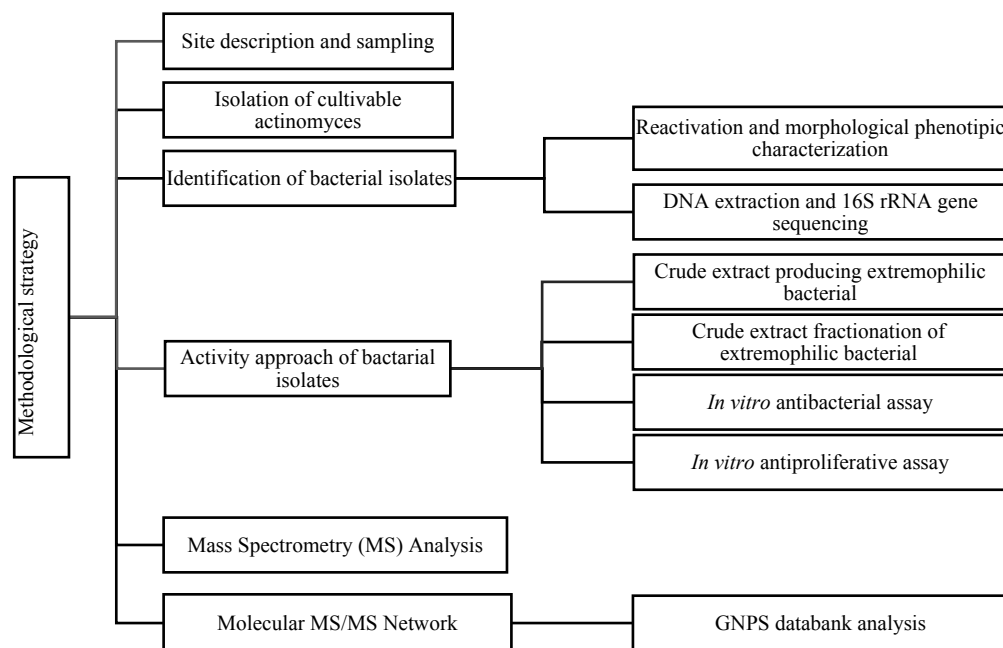
Fig. 2. Flowchart depicting the methodological strategy adopted in this study.

125

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

136   and stored in a refrigerated chamber at -20 ° C to the transport to the Microbial Resources

137   Division of the Pluridisciplinary Center for Chemical, Biological and Agricultural Research

138   (CPQBA) in August 2015.

139

140   **2.3 Identification of bacterial isolates**

141   **2.3.1   Reactivation and morphological phenotypic characterization of bacterial**

142   **strains**

143   The reactivation of the isolates was carried out in R2A broth (Difco ref. 234000),

144   supplemented with artificial seawater (ASW) NaCl 5% (0.1 g potassium bromide, 23.48 g

145   sodium chloride, 10.61 g of magnesium chloride, 1.47 g of calcium chloride, 0.66 g of

146   potassium chloride, 0.04 g of strontium chloride, 3.92 g of sodium sulfate, 0.19 g of sodium

147   bicarbonate, 0.03 g of boric acid all in 1 L of distilled water) at 28 °C for 28 days, the colonies

148   were observed under a microscope and stereoscope for macro morphological characterization

149   and after Gram staining, the cells were observed under an optical microscope. The bacteria

150   were preserved in 20% glycerol and kept at -80 °C.

151

152   **2.3.2   DNA extraction and 16S rRNA gene sequencing**

153   An isolated colony was used for extraction of genomic DNA, according to the method

154   described by Pospiech and Neuman, 1995, with some modifications. Amplification of the

155   16S ribosomal RNA gene was done by means of a polymerase chain reaction (PCR) using

156   the pair of primers p10f (5' GAG TTT GAT CCT GGC TCA G 3') and p1401r (5' CGG

157   TGT GTA CAA GGC CCG GGA ACG 3') or p1492r (5'- ACC TTG TTA CGA CTT 3')

158   (Lane et al. 1985) homologous to conserved regions of the bacterial 16S ribosomal RNA

159   gene. Conditions used in the amplification reaction were: 2.5 μL of 10X buffer (without

160   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

161   μM), 0.2 μL of Taq DNA polymerase (Invitrogen) and 5.0 μL of the DNA template (50-100

162   ng/ μL) and deionized and sterilized water by filling the volume to 25 μL of reaction. The

163   PCR amplification was an initial denaturation step of 5 min at 95°C, followed by 30 cycles

164   of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, 3 min at 72°C for extension

165   and 3 min at 72°C for final extension in an Eppendorf thermal cycler. The obtained products

166   were analyzed and visualized in agarose gel electrophoresis, purified with mini columns

167   (GFX PCR DNA & gel band purification kit, GE Healthcare), and sequenced in ABI3500XL

168   Series automatic sequencer (Applied Biosystems). Sequencing reactions were performed

169   with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems)

170   according to the manufacturer's specifications.

171   Partial sequences of the 16S ribosomal RNA gene obtained from each isolate were assembled

172   into a contig and then compared to the sequences of organisms represented in EZBioCloud

173   16S Database (https://www.ezbiocloud.net/) using the "Identify" service (Yoon et al. 2017),

174   and species assignment were based on closest hits (Jeon et al. 2014). 16S ribosomal RNA

175   gene sequences retrieved from the database and related to the unknown organism gene were

176   selected for alignment in the Clustal X program (Thompson et al. 1997), and phylogenetic

177   analyzes were performed using the Mega version 6.0 program (Tamura et al. 2013). The

178   evolutionary distance matrix was calculated with the model of Kimura-2 parameters

179   (Kimura, 1980), and the phylogenetic tree constructed from the evolutionary distances

180   calculated by the Neighbor-Joining method (Saitou and Nei, 1987), with bootstrap values

181   from 1000 resampling.

182

183   **2.4 Activity approach of bacterial isolates**

184   **2.4.1   Crude extract producing extremophilic bacterial**

185   Secondary metabolites of bacterial isolates were extracted with ethyl acetate. A pre-inoculum

186   of the bacteria was performed in 5.0 mL of R2A Broth medium (Himedia ref. 1687) with

187   AWS NaCl 5% and incubated at 28 °C for 5 to 7 days. After the growth of the culture, the

188   total volume was transferred to an Erlenmeyer containing 500.0 mL of the same culture

189   medium and incubated for 7 days under agitation at 150 rpm, followed by 23 days without

190   agitation. After this period, 500.0 mL of ethyl acetate were added to the medium containing

191   the bacterial growth and ruptured in ultra turrax basic (IKA ref. 02H2063.08.CC) at 7,000

192   rpm for 15 min. The organic fraction was obtained and the crude extract was concentrated in

193   the rotary evaporator (R-215 Buchi), under vacuum and temperature of 40 °C, until the

194   solvent was completely dried and stored at 4°C. The crude extracts of three strains

195   representatives were taken for antimicrobial and antiproliferative activity tests.

196   Simultaneously, the crude extracts also were analyzed by liquid chromatography coupled to

197   the mass spectrometer (Versamax, molecular manufacturer Devices).

198

199   **2.4.2   Crude extract fractionation of the extremophilic bacterial.**

200   The crude extracts of the isolates were fractionated using a vacuum chromatographic column

201   with C18. The initial crude extract was solubilized in methanol. The mobile phases used were

202   fraction 1 water (H_2O); fraction 2 water: methanol (H_2O: MeOH 1:1 v/v); fraction 3 methanol

203   (MeOH); fraction 4 MeOH: ethylacetate (EtOAc) (1:1 v/v); fraction 5 EtOAc 100%; fraction

204   6 EtOAc: Glacial acetic acid 1%. These fractions were dried in rotary evaporator at 45 °C,

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

206

207   2.4.3   *In vitro* antibacterial activity assay

208   Three crude bacterial extracts (M-92, B-81 and B-146) were tested as antimicrobial producers

209   using the minimum inhibitory concentration (MIC) assay, following the protocol reported by

210   Siddharth and Rai 2018. The crude extracts partially diluted in 1% Dimethylsulfoxide

211   (DMSO) 1 mg/mL – 3.9 µg/mL, and sterile broth were added into pre-coated microbial

212   cultures, completed a total volume of 200 µL. The plate was incubated at 37 °C and room

213   temperature; the lowest concentration of extract, which completely inhibited the bacterial

214   growth was considered as MIC. Each biological assay was performed in triplicate. The

215   pathogenic bacteria used in this test were *Escherichia coli* ATCC 10536, *Staphylococcus*

216   *aureus* ATCC 6538, and *Acinetobacter baumannii* with code AC-972, the strain was not

217   acquired prospectively, the source of origin comes from the bank of MDRs with pneumonia

218   from the UCI of the Hospital Regional Lambayeque, details such as patient data linked to the

219   sample were anonymized in order to obtain access

220

221   2.4.4   *In vitro* antiproliferative activity assay

222   This assay aimed to detect anticancer activities by evaluating antiproliferative action in

223   human tumor cells (Monks et al. 1991). *In vitro* tests with the crude extract of *Streptomyces*

224   sp. M-92, B-81, and B-146 on human tumor cell lines of different origins and a non-tumor

225   cell line. Human tumor cell lines U251 (glioma), UACC-62 (melanoma), MCF-7 (breast),

226   NCI-ADR/RES (ovarian expressing phenotype with multiple drugs resistance), 786-0

227   (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), K562

228   (leukemia) and a non-tumor cell line HaCaT (keratinocyte) were obtained from the National

229   Cancer Institute Frederick, Molecules (MD, USA). Stock cultures were grown in medium

230   containing 5 mL of RPMI 1640 (GIBCO BRL, Gaithers-Burg, MD, USA) supplemented with

231   5 % fetal bovine serum (FBS, GIBCO) at 37 °C with 5% CO_2. Penicillin: streptomycin (1000

232   μg·L−1:1000 U·L−1, 1 mL·L−1) was added to the experimental cultures. Cells in 96-well

233   plates (100 μL cells well−1) were exposed to the extracts in DMSO (Sigma-Aldrich) /RPMI

234   (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

235   concentration (0.2% in higher concentration) did not affect cell viability. Before (T_0) and

236   after (T_1) sample application, cells were fixed with 50% trichloroacetic acid (Merck), and

237   cell proliferation was determined by the spectrophotometric quantification (540 nm) of

238   cellular protein content using sulforhodamine B assay. Using the concentration-response

239   curve for each cell line, the values of the sample concentration required to produce total

240   growth inhibition or cytostatic effect (TGI) were determined through non-linear regression

241   analysis using software ORIGIN 8.6® (OriginLab Corporation, Northampton, MA, USA).

242

243   **2.5 Mass Spectrometry analysis**

244   *Streptomyces sp.* B-81 extract was resuspended in 1 mL of methanol (HPLC grade) and 100

245   μL were diluted in 900 μL of methanol. The final solution was filtered through 0.22 μm into

246   vials. Ultra-high pressure liquid chromatography-mass spectrometry (UHPLC-MS) analyses

247   were performed in a Thermo Scientific QExactive® Hybrid Quadrupole-Orbitrap Mass

248   Spectrometer. Parameters: positive mode, capillary voltage at +3.5 kV; capillary temperature

249   at 250 °C; S-lens of 50 V and *m/z* range of 133.40-2000.00. Tandem mass spectrometry

250   (MS/MS) was performed using normalized collision energy (NCE) of 30 eV and 5 precursors

13

251   per cycle were selected. Stationary phase: Thermo Scientific Accucore C18 2.6 µm (2.1 mm

252   x 100 mm) column. Mobile phase: 0.1% formic acid (A) and acetonitrile (B). Eluent profile

253   (A: B) 0-10 min, gradient from 95:5 up to 2:98; held for 5 min; 15-16.2 min gradient up to

254   95:5; held for 8.8 min. Flow rate: 0.2 mL min^{-1}. The injection volume of the samples was 3

255   µL. Operation and spectra analyses were conducted using Xcalibur software (version 3.0.63)

256   developed by Thermo Fisher Scientific.

257

258   **2.6 Molecular MS/MS network**

259   A molecular network for *Streptomyces sp*. B-81 was created using the online workflow at

260   GNPS (https://gnps.ucsd.edu/). The data were filtered by removing all MS/MS peaks within

261   ± 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top

262   6 peaks in the ± 50 Da window throughout the spectrum. The data was then clustered with

263   MS-Cluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of

264   0.02 Da to create consensus spectra. A network was then created where edges were filtered

265   to have a cosine score above 0.5 and more than 5 matched peaks. Further edges between two

266   nodes were kept in the network only if each of the nodes appeared in each other's respective

267   top 10 most similar nodes. The spectra in the network were then searched against GNPS'

268   spectral libraries. The library spectra were filtered in the same manner as the input data. All

269   matches kept between network spectra and library spectra were required to have a score

270   above 0.5 and at least 5 matched peaks (Wang et al. 2016).

271

272 # 3. Results and discussion

273 **3.1 Isolation, identification and selective of bacterial species from the northern**

274 **saline lagoons of Perú**

275 In total, 166 pure cultures showing different colony morphologies were obtained grown on

276 R2A medium from saline water (A), water and sediment (AES), and sediment (SE). The

277 morphological characteristics such as aerial mycelium, the morphological spore mass color,

278 pigmentation of vegetative or substrate mycelium, and the production of diffusible pigment

279 were used to classify 42 filamentous and 8 non filamentous to the genera *Streptomyces* 39

280 (78%), *Pseudonocardia* 3 (6%), *Staphylococcus* 4 (8%), *Bacillus* 2 (4%), and *Pseudomonas*

281 2 (4%) in (Table 2).

282

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

284 **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*

285

286 The isolates were regrouped into 28 isolates due to their similar phenotypic characteristics

287 and were identified based on the sequencing and alignment, from the 16S rRNA gene

288 analyzes. They belong to the genus *Streptomyces* 27 isolates and 1 *Pseudonocardia* isolate,

289 and 23 isolates showed a 16S rRNA gene sequence similarity below the 98.7% threshold for

290 proposing as novel species (Table 3).

["

|				
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

296

297 Therefore, these strains should be designed for further taxonomic and analytical chemistry

298 analyses to confirm their novelty at species rank and as a source of novel chemical entities.

299 Two isolates were identified in lagoon 1 of the Morrope salt flats, and according to the

300 EZBioCloud the isolate M-92 presented similarity with *Streptomyces luteus* TRM 45540

301 (99.16%), while the isolate M-142 presented similarity with *Streptomyces labedae* NBRC

302 15864 (97.58%). In lagoon 2 of the Morrope salt flats, 13 isolates were identified, of which

303 M-239 and M-239.2 were similar to *S. luteus* TRM 45540 (99.19%), the isolates M-261 and

304 M-274.2 presented similar to *S. griseoincarnatus* LMG 19316 (97.58%), the isolate M-262

305 showed similarity to *S. griseorubens* NBRC 12780 (97.57%), the isolate M-266 was similar

306 to *S. variabilis* NBRC 12825 (97.58%), the isolates M-270 and M- 289.1 were similar to *S.*

307 *pactum* NBRC 13433 (97.98%), the isolate M-290 presented similar to *S. hyderabadensis*

308 OU-40 (98.06%), and finally four isolates, M-275, M-282.2, M-285 and M- 290.1 were

309 similar to *S. olivaceus* NRRL B-3009 (97.98%). From the lagoon 3, also belongs to the

310 Morrope salt flats, it was identified one isolate M-323 similar to *S. olivaceus* NRRL B-3009

311 (97.98 %). Finally, from lagoon 4, belongs to the Bayovar salt flats, 12 isolates were

17

312   identified, four isolates were similar to *S. olivaceus* NRRL B-3009, B-81 (99.93%0, B-144.2

313   (97.98%), B-146 (99.84%), and B-244 (97.98%). Also, in the lagoon 4 the isolate B-93, was

314   similar to *S. violascens* ISP 5183 (97.37%), and other four isolates presented similar *S.*

315   *griseoincarnatus* LMG 19316, B-93.3, B-95, B-236, B-265 (97.58%), the isolate B-95.1 was

316   similar to *S. labedae* NBRC 15864 (97.58%), and the isolate B-379 was similar to *S.*

317   *hydrogenans* NBRC 13475 (97.27%). Besides Streptomyces, another of the actinobacteria

318   genus was recovered to the Bayovar salt flats. The isolate B-236.1 was similar to

319   *Pseudonocardia antimicrobica* YIM 63235 (97.27%) and our work is the first to report this

320   genus present in the Bayovar salt flats (Table 3).

321   In the Morrope salt flats, eight different groups of *Streptomyces* were identified, whereas in

322   the Bayovar saline only five groups of *Streptomyces* were recovered, however, a different

323   genus like *Pseudonocardia*, whichhas been reported by Zhang et al. (2016) as producers of

324   γ-Butyrolactones that previously had only been reported for Streptomyces genera and in

325   plants was recovered in this study (Table 3). Our culture-based approach using a pre-

326   enrichment to decrease the bacterial load agrees with the reports described in the literature,

327   in which the isolation of halophilic bacteria reveals low species richness and the dominance

328   of *Streptomyces* genus Parthasarathi et al. (2012). Ballav et al. (2015) reported *Streptomyces*

329   spp. as the most predominant group contributing to 46% of the total isolates in crystallizer

330   pond sediments of Ribandar saltern, Goa, India. In this study the isolation was performed

331   with three different types of culture media and four different concentrations of salt because

332   in lagoons were found the concentration of salts between 0-300 psu units (Ballav et al. 2015).

333   Cortes et al. (2019) in the Salar de Huasco report similar results, they were isolated

334   *Streptomyces* (86%), *Nocardiopsis*, (9%), *Micromonospora* (3%), *Bacillu*s (1%) and

18

335 *Pseudomonas* (1%).

336 The phylogenetic analysis based on their nearly complete 16S rRNA gene sequences (>1206

337 bp) was constructed with the isolates M-92, B-81 and B-146 because them presented

338 antibacterial and antiproliferative activities. The phylogenetic tree showed clades well

339 supported to the three isolates and the reference sequences (Figure 3).

340
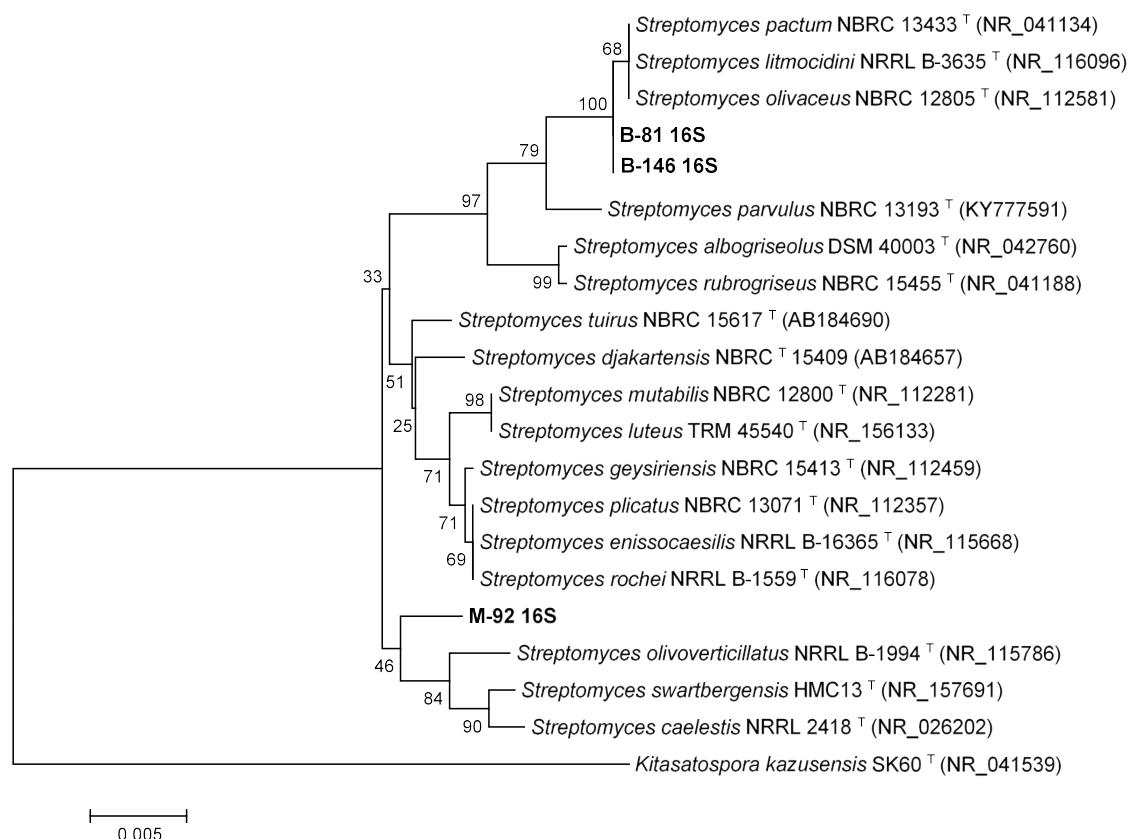
341 **Fig 3. Phylogenetic tree based on 16S rRNA gene sequences (1206 positions in the final**

342 **dataset) inferred using the neighbour-joining method in MEGA7, the evolutionary**

343 **distances were computed using the Kimura 2-parameter method, showing the**

344 **phylogenetic positions of different saline isolates and type strains within the genus**

345 **Streptomyces and Pseudonocardia. Numbers at branching points refer to percentages**

19

346 **of bootstrap values from 1000 replicates. The scale bar indicates 0.02 indicated**

347 **substitutions per nucleotide. *Kitasospora kazusensis* SK60^T (NR_041539) was used as**

348 **outgroup.**

349 Isolate M-92 forms a distinct branch closely related to the type strain of *Streptomyces*

350 *olivoverticulatus* NRRL-1994^T, *Streptomyces swartbergensis* HMC13^T, and *Streptomyces*

351 *caelestis* NRRL-2418^T. Isolates B-81 and B-146 form a well-supported sub-clade closely

352 associated to *Streptomyces olivaceus* NBRC 12805^T, *Streptomyces litmocidini* NRRL B-

353 3635^T, and *Streptomyces pactum* NBRC 13433^T (Figure 3). The length of the branch of all

354 *Streptomyces* isolates in the phylogenetic tree and the assignation to these isolates to

355 completely different clades from each other, highlight the divergence of them from their

356 closely related neighbors. Further studies need to be performed to confirm the right

357 affiliations of these isolates to the novel species within the evolutionary radiation of the genus

358 *Streptomyces*.

359

360 **3.2 Antibacterial activity Screening**

361 Minimum Inhibitory Concentration (MIC) assay was used to screen antibacterial metabolites

362 producer strains against the indicator strains *Escherichia coli* ATCC 10536, *Staphylococcus*

363 *aureus* ATCC 6538, and *Acinetobacter baumannii* AC-972 MDR; the crude extracts tested

364 from the three extremophilic bacterial reported positive results against the pathogenic

365 bacteria tested. The antimicrobial activity of the crude extract of *Streptomyces* sp. B-81 strain

366 present the highest inhibitory activity with the lowest MIC with 7.82 μg/mL against the three

367 pathogens, followed by *Streptomyces* sp. M-92 showed MIC of 7.82 μg/mL against *E. coli*

368 ATCC 10536 and *S. aureus ATCC 6538*, and 15.63 μg/mL against *A. baumannii AC-972*

369   MDR. *Streptomyces* sp. B-146 showed MIC of 15.63 µg/mL against the pathogens tested. It

370   is important to highlight that all crude extracts of the strains presented low MIC in

371   comparison with the reference antibiotic (Table 4).

372

373   **Table 4. Minimum inhibitory concentration MIC antibacterial of three *Streptomyces* sp.**

374   **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

375

376   Similar results were reported in a marine *Streptomyces* sp. S2A with MIC 31.25 µg/mL

377   against *Klebsiella pneumoniae*, 15.62 µg/mL against *Staphylococcus epidermidis*,

378   *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli* and *Micrococcus luteus* with 7.8

379   µg/mL (Saket Siddharth and Ravishankar R. Vittal, 2018). The crude extract of the

380   *Streptomyces* sp. YBQ59 strain against nine pathogens, obtaining MIC results between 10.5

381   to 22.5 µg/mL, their results being similar to those we obtained (Vu. H et al. 2018).

382

383   **3.3 Antiproliferative activity Screening**

384   The antiproliferative activity of the crude extracts of *Streptomyces* sp. M-92, B-81, and B-

385   146 against three human tumor cell lines were expressed as TGI (Total Growth Inhibition-

386   concentration that inhibited cell growth by 100%) (Table 5).

387

388 **Table 5. Antiproliferative activity of three Streptomyces extracts against human**

389 **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

390 Human tumor cell lines: U251 (glioma), MCF-7 (breast), NCI-H460 (lung of type non-small cells)
391 [a, b] Doxorubicin used as positive control;
392 [c] TGI Concentration that totally inhibited cell growth
393

394 According to Abreu et al. (2014), TGI values higher than 50 µg/mL represent inactive

395 samples. In comparison to the others *Streptomyces* tested and doxorrubicin, *Streptomyces* sp.

396 B-81 shown the best result against glioma (TGI =0,57 µg/mL), lung cancer (TGI =0,61

397 µg/mL) and breast cancer (TGI =0,80 µg/mL). Due to the result obtained with the crude

398 extract of *Streptomyces* sp. B-81 strain, the extracts from three different media were selected

399 for further fractionation, to have access to the metabolic profile. It was possible to verify that

400 the highest activity detected was in the extracts from the fermentation of *Streptomyces* sp. B-

401 81 in the R2A medium (Table 5), which inhibited the nine different types of tumor cell lines.

402 However, it is observed that the fractionation decreases the antitumor activity presented in

403 the crude extract and the putative active compounds are recovered in fractions 3 and 4.

404 Fraction 3 seemed to contain antiproliferative substances in lower concentration and was able

405 to inhibit U251 (glioma) (TGI = 38.3 µg/mL), OVCAR-03 (ovary) (TGI = 62.1 µg/mL), and

406 K562 (leukemia) (TGI = 81.5 µg/mL), and fraction 4 inhibited U251 (glioma) (TGI =

407 73.5 µg/mL) and UACC-62 (melanoma) (TGI = 89,4 µg/mL) (Table 6).

408 **Table 6. Antiproliferative evaluation of isolate *Streptomyces* sp. B-81 fermented in three**

409 **different medium and fractions of the R2A medium against nine tumors and one non-**

410 **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	*	*	*	*	*	*	*	*	*

411 B-81 NB: *Streptomyces sp.* Crude extract in nutrient broth growth medium. B-81 ISP2: *Streptomyces sp.* Crude
412 extract in yeast extract-malt. B-81 R2A: *Streptomyces sp.* Crude extract in R2A growth medium. B-81 R2A
413 fractions: 1 = H$_2$0; 2 = MeOH-H$_2$O; 3 = MeOH; 4 = MeOH-EtOAc; 5 = 100% EtOAc; 6 = EtOAc -acid: Human
414 tumor lines: 2 = U251 (glioma), u = UACC-62 (melanoma); m = MCF-7 (breast); a = NCI-ADR / RES (ovary
415 with multiple drug resistance phenotype); 7 = 786-O (kidney adenocarcinoma); 4 = NCI-H460 (lung, non-small
416 cell type); o = OVCAR-03 (ovary); h = HT-29 (colon); k = K562 (leukemia). Human non-tumor lineage: q =
417 HaCaT (keratinocyte).
418 Doxorubicin (Reference chemotherapy).
419 TGI: Total Growth Inhibition- Concentration necessary to completely inhibit cell proliferation.
420 * TGI >250 µg/mL
421

422 It is common to find this antiproliferative activity in the *Streptomyces* genus due to its

423 metabolic and genetic capacity to produce secondary metabolites (Tan and Liu, 2017).

424 Additionally, actinomycetes isolated from marine environments have a greater capacity to

425 express these metabolites in response to the pressure of environmental selection to which

23

426   they have been subjected throughout the evolutionary history of aquatic organisms (Lam,

427   2006). This pressure has generated high specificity and a complex three-dimensional

428   conformation of the compounds to act in the marine environment (Song et al, 2018). In this

429   particular case, the geographical isolation, the geological conditions of formation of the

430   saline lagoon and the extreme environmental conditions generate a greater selection

431   environmental pressure, which makes it possible to observe antibiotic and antiproliferative

432   activity in the isolates analyzed.

433   The anticancer potential of halophilic actinomycetes has been extensively studied. Secondary

434   metabolites Salternamides A-D isolated from halophilic actinomycetes of saline on the island

435   of Shinui (Republic of Korea), have potential cytotoxicity against the human colon cancer

436   cell line (HCT116) and gastric cancer cell line (SNU638) (Kim et al. 2015). Another

437   moderately halophilic *Streptomyces sp*. W1 with number accession GenBank JN187420.1,

438   isolated from Weihai Solar Saltern in China, has potent cytotoxicity against human lung

439   cancer cell line (A549) with IC_{50} 78.6 µM; human cervical epithelial cell line (HeLa) IC_{50}

440   56.6 µM, human liver cancer cell line (BEL-7402) with IC_{50} 47.1 µM, and human colon

441   cancer cell line (HT-29) with IC_{50} 94.3 µM (Liu et al. 2013). Four compounds called

442   shellmicin A-D isolated from the *Streptomyces* sp. shell-016 were described by (Yong Han

443   et al. 2020) and studied concerning cytotoxicity against five human tumor cells lines: non-

444   small cell lung cancer (H1299, ATCC CRL-5803), malignant melanoma (A375, ATCC CRL-

445   1619), hepatocellular carcinoma (HepG2, ATCC HB-8065), colorectal adenocarcinoma

446   (HT29 ATCC HTB-38) and breast cancer (HCC1937, ATCC CRL-2336). The study

447   indicated that the compounds Shellmicin A, B and D showed greater cytotoxicity than

448   Shellmicin C compound, with an IC_{50} ranging from 0.69 µM to 3.11 µM at 72 h. An

449   interesting fact was that shellmicin C and D are a pair of stereoisomers and their biological

450   activity was significantly different. It is worth mentioning that in our study we determined

451   that the activity of the crude extract was better than that of the fractions, the same ones that

452   presented a different activity, therefore, we can deduce that we would be facing compounds

453   with synergism.

454   An actinomycete strain designated as *Streptomyces* sp. KML-2 isolated from a saline soil

455   mine in Khewra, Pakistan, showed antitumor activity against Hela cells (cervical cancer) and

456   MCF-7 (breast cancer) cell lines with IC_{50} values of 8.9 and 7.8 µg/mL respectively in the

457   initial screening, then they carried out a higher production (20 L) for the purification of the

458   compounds, confirming a wonderful anti-tumor potential against MCF-7 cells with an IC_{50}

459   value of only 0.97 µg /mL (Usman et al. 2015). This study also revealed that the Khewra salt

460   mine from which the KML-2 strain was isolated is a powerful ecological niche with

461   inimitable strain diversity that has yet to be discovered, a fact that reinforces the approach of

462   our study in the search for new compounds with biotechnological activity.

463

464   **3.4 Secondary Metabolite Analysis of *Streptomyces sp*. B-81 isolate.**

465   The crude extract obtained from *Streptomyces* sp. B-81 was investigated through UHPLC-

466   MS/MS analyses and demonstrated a broad profile of biomolecules (Fig. S1A). Furthermore,

467   a metabolite screening in the GNPS platform was performed and molecular networking

468   revealed two clusters (A and B) that exhibited secondary metabolites produced by

469   *Streptomyces sp*. B-81 extract (pink) (Fig. 4).
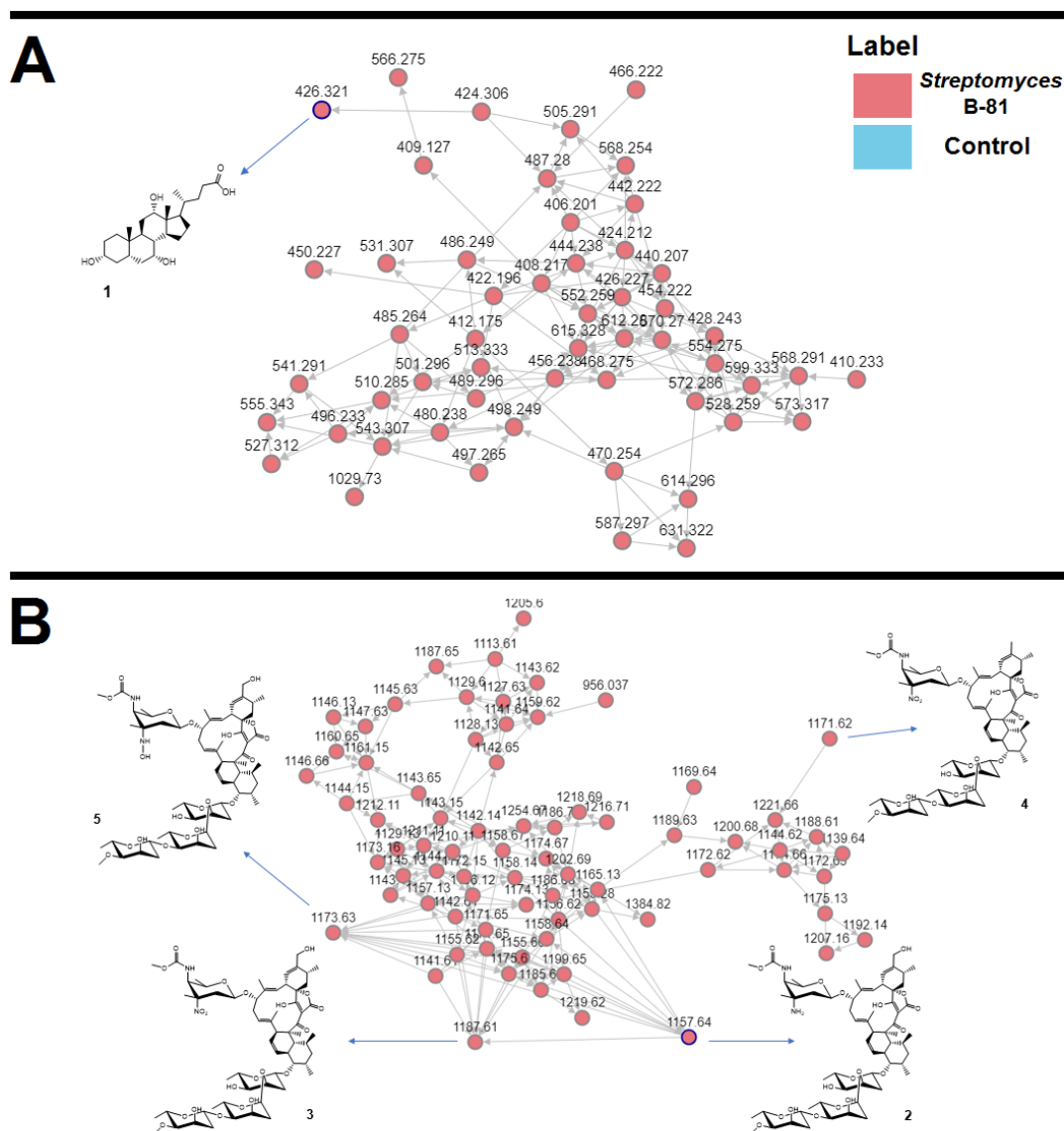
Fig 4. Molecular networking obtained for *Streptomyces* sp. B-81 extract. Cholic acid and derivatives were grouped in cluster A, and lobophorins were grouped in cluster B. Nodes circled in blue indicate molecules identified by comparison with the GNPS platform database.

476 Metabolites were identified as a hit in the GNPS database or manually identified by accurate

477 mass analyses, which showed mass errors below 5 ppm (Table 7).

478

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

480 **extract.**

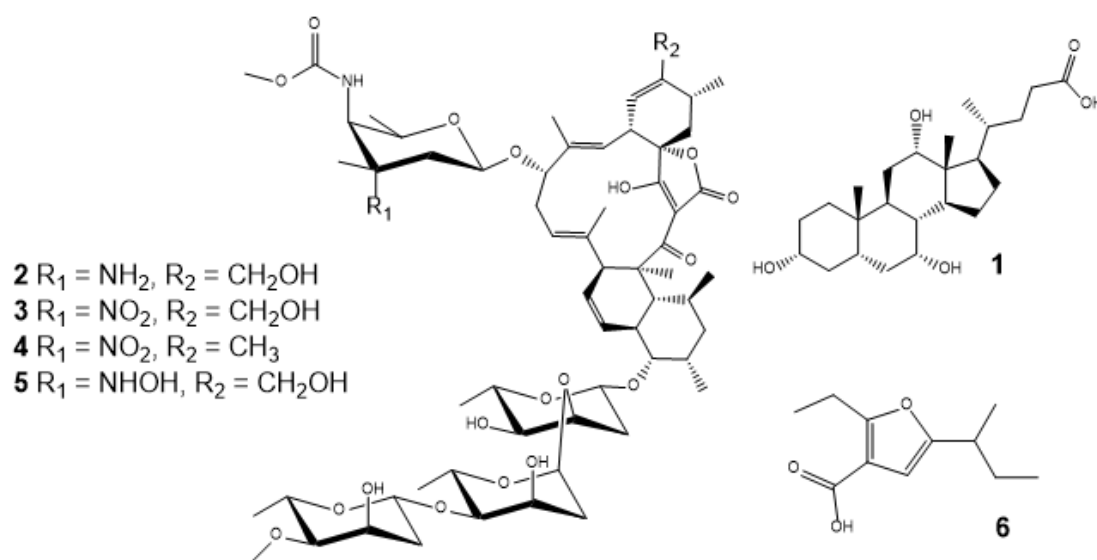| Compound	Ion formula	Calculated *m/z*	Experimental *m/z*	Error (ppm)
Cholic acid	$C_{24}H_{44}NO_5$	426.3219	426.3212	-1.6
Lobophorin A	$C_{61}H_{93}N_2O_{19}$	1157.6372	1157.6373	0.1
Lobophorin B	$C_{61}H_{91}N_2O_{21}$	1187.6114	1187.6108	-0.5
Lobophorin E	$C_{61}H_{91}N_2O_{20}$	1171.6165	1171.6160	-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

481

482 The observed signals corresponded to cholic acid (1), lobophorin A (2), lobophorin B (3),

483 lobophorin E (4) and lobophorin K (5).

484 In cluster A, GNPS database indicated the presence of cholic acid in the *Streptomyces* sp. B-

485 81 extract (Fig. S2 and S3). In the molecular networking, each MS/MS spectra are

486 represented by nodes, which are grouped in clusters based on their fragmentation pattern

487 similarity. Thus, compounds of the same class of molecules are grouped in the same cluster

488 (Wang et al. 2016). As observed in Figure 4, the cholic acid cluster is composed by a range

489 of nodes, suggesting the presence of other analogues. Cholic acid and other bile acids have

490 been reported as secondary metabolites of specific members of the genus *Streptomyces* and

491 *Myroides* (Kim et al. 2007). In the literature, cholic acid-derivatives and bile acids are known

492 as antimicrobials agents, displaying activity against gram-negative and positive bacteria or

493   improving the antimicrobial effect of antibiotics (Schimidt et al. 2001, Rasras et al 2010,

494   Darkoh et al. 2010, Savage et al. 2000).

495   In cluster B, GNPS database indicated the production of lobophorin A by *Streptomyces* sp.

496   B-81 strain (Fig. S4 and S5). Also, MS/MS profile of compound **2** presented *m/z* 883.49 and

497   753.43 (Fig. S5), related to the loss of the sugar units in the structure, according with the

498   literature (Nguyen et al. 2020). Similar as observed in cluster A, cluster B also exhibited

499   other lobophorin compounds such as lobophorins B, E and K that were identified based on

500   their accurate masses and fragmentation profiles with typical fragments at *m/z* 184.09,

501   157.09, 108.08 and 97.06 (Fig. S6-S8). Lobophorins were discovered by Jiang et. al. (1999)

502   in the expedition to Belize on board of the Columbus research ship. In the occasion, a new

503   strain of actinomycete, named as # CNC-837, was isolated from the surface of a brown algae

504   from the Caribbean called *Lobophora variegata*, and was reported to produce lobophorins A

505   and B, potent anti-inflammatory agents (Jiang et al. 1999). Besides lobophorins A and B,

506   other compounds of this class have been reported in the literature. Lobophorins C and D have

507   similar structures to A and B, while Lobophorin C exhibited potent cytotoxic activity against

508   human liver cancer cells, lobophorin D displayed significant inhibitory effect on human

509   breast cancer cells (Wei et al. 2011). Lobophorins E and F were reported by Niu et al. 2011,

510   that also described antibacterial and cytotoxic activities for lobophorin F. In other study, Pan

511   et al. 2013 reported lobophorins H and I and described that lobophorin H exhibited

512   antibacterial activities against *Bacillus subtilis*. Most recent, Lobophorin K was reported

513   (Braña et al. 2017) and also displays antimicrobial and cytotoxic activities.

514 Besides cholic acid and lobophorins, a furan-type compound (6) were also detected in the

515 *Streptomyces sp*. B-81 extract (Fig. S9). The structures of the metabolites **1-6** are shown in

516 Fig. 5.

2 R_1 = NH$_2$, R_2 = CH$_2$OH
3 R_1 = NO$_2$, R_2 = CH$_2$OH
4 R_1 = NO$_2$, R_2 = CH$_3$
5 R_1 = NHOH, R_2 = CH$_2$OH

517

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

519

520 Compound 6 exhibited fragments at m/z 168.07, 167.07 and 153.05, in agreement with the

521 literature (Nguyen et al. 2020). Compound 6 was reported as a secondary metabolite of the

522 *Streptomyces sp*. VN1 strain, a microorganism isolated of sea sediment from a coastal region

523 of Viet Nam (Nguyen et al. 2020). In addition, *Streptomyces sp*. VN1 was also described to

524 produce lobophorin A (Nguyen et al. 2020). Nguyen et al 2020, reported that compound 6

525 displayed cytotoxic activity, *in vitro*, against 5 types of tumor cell lines. Furthermore, Wang

526 et al. 2008 reported that other furan compound named HS071, produced by *Streptomyces sp*.

527 HS-HY-071, displayed anticancer activity.

528

529   Our data deserves further investigation in order to correlate the antimicrobial and cytotoxic

530   activities, observed for the *Streptomyces* sp. B-81 extract, to the bile acids, lobophorins and

531   furan compounds. In addition, the extremophilic environments, in this particular case the

532   Salinas de Bayovar, are prolific sources of microorganisms, such as unique *Streptomyces*.

533   Due the extreme conditions, these microorganisms produce different compounds in order to

534   adapt to survive in the environment, which makes *Streptomyces* sp. B-81 an attractive source

535   of bioactive compounds.

536

Conclusion

537

538   The present study was successful in determining the diversity and bioactive potential of the

539   actinobacterial isolates present in this type of environment (Mórrope and Bayovar salt flats)

540   located in northern Peru, which are environments that had not been explored for this type of

541   study. this being the first study. Furthermore, it has been found that the isolates of

542   actinobacetria *Streptomyces sp*. M-92, B-146 and B-81 may be new species, their importance

543   for these isolates lies in their antibacterial and antiproliferative potential, being the most

544   promising for their best activity *Streptomyces sp*. B-81. Six biomolecules (colic acid,

545   Lobophorin A, B, E and K in addition to a sixth compound) were detected in this cultivable

546   actinobacterial isolated from Bayovar's salt flats, but not yet named from the cured base

547   GNPS. With this study it is determined that the actinobacteria that live in these environmental

548   conditions present an enormous wealth of microorganisms that are new species that produce

549   new and biologically active compounds and contribute to the study of extreme environments

550   such as the saline lagoons of northern Peru with antibacterial potential. and antiproliferative.

551   Further studies will be carried out in the identification of these molecules in order to purify

552   and characterize them since this can result in the economic production of bioactive

553   compounds for future pharmaceutical applications.

554

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566

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