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Antibacterial activity of Lake Urmia derived-*Halomonas*

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Abstract

Isolation of new microbial species from extreme environments is one of the most efficient approaches for the development of novel bioactive metabolites. The aim of the present study was to explore the pharmaceutical bacterial resources from the water and sediments of hypersaline Lake Urmia. Using different culture conditions and media led to the isolation of 20 bacterial strains. Halophilic bacteria were screened for the production of antibacterial agent against multi-drug resistant strains of *Escherichia coli* through agar well diffusion assay. Halophilic bacteria DNA extraction was done by boiling method. The results showed that two *Halomonas* strains, LUH16 and LUH20 identified by analysis of 16S rRNA gene sequences were the potent producers of antimicrobial metabolites against various strains of *E. coli*. Furthermore, gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of eight secondary metabolites with the relevant antimicrobial properties. Our findings led us to focus on *Halomonas* strains as potent producers of antimicrobial compound that might be an alternative against antibiotic-resistant pathogens such as pathogenic *Escherichia coli*.

Introduction

The use of antibiotics for the treatment and prevention of bacterial infections in poultry farms has led to the increase of resistant bacteria. This issue has raised concerns regarding the potential impact of antibiotics on human health due to the emergence of antibiotic-resistant microorganisms. *Escherichia coli* have an important role within resistant bacteria populations being widely used as a bio-indicator of antimicrobial resistance and being pathogenic to humans and animals.[1]

Antimicrobial resistance accounts for at least 700,000 deaths annually in the world, and its mortality will be compared to those of cancers and cardiovascular diseases by 2050.[2] Therefore, there is an urgent need to find new bioactive compounds with clinical significance.[3] In recent years, different bacteria from intact environments, such as hypersaline marine habitats, have attracted the interests of researchers as a source of novel bioactive compounds.[4-6] With this perspective, Lake Urmia in Northwest of Iran is a pristine and suitable area for the exploration of antibiotic-producing microorganisms. Lake Urmia is one of the largest hypersaline lakes in the world with an altitude of 1,250 m.[7] Due to considerable climate changes, low rate, and causeway construction, which divides the lake into south and north arms, NaCl concentration of Lake Urmia has raised to more than 300 gL^{-1} during recent years approximately similar to that of the Dead Sea.[7-10] During the last decade, with a growing interest in extremophiles, a large number of halophiles particularly species belonging to the *Halomonas* genus have been isolated from the different parts of Lake Urmia in order to determine the diversity and potential uses of these bacteria.[11-13] However, exploring this region as a source of antimicrobial compounds of bacterial origin has not been previously described.

In the present study, we isolated halophilic strains from sediments of Lake Urmia, tested their antimicrobial activity against multidrug-resistant strains of avian pathogenic *E. coli* and determined antimicrobial compounds by gas chromatography-mass spectrometry (GC-MS) analysis.

Materials and Methods

Site description and sampling. Lake Urmia (37° 32' N, 045° 43' E) which includes 7.00% of the total surface water in Iran, has been designated as a national park and

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protected biosphere region by the UNESCO.[7,14] In this study, sampling was performed from different sites of the north part of the lake namely Aq Gonbad, Burachalo, Kushk, Sharafkhaneh port, Sheikh Vali, Qharabagh, Govarchin Ghalea, Bari, Gol Tappeh, Khaneshan. The information for the samples is listed in the Table 1. Samples of sediments (100 g) were gathered up from March to May 2019. Sediments samples at 15.00 cm depth below the surface aseptically were collected in the sterile plastic bottles, delivered to the laboratory and stored at 4.00 ˚C for further assay. Physical parameters were analyzed *in situ* using a Hach pH meter and thermometer (Biolab, Barcelona, Spain).[15]

Isolation, screening and culture conditions of strains. Following collection, samples were serially diluted before plating on different media. One gram of sediment samples was suspended in 100 mL of sterile water and placed on rotary (IKA, Staufen, Germany) at 120 rpm for 30 min. Serial dilutions of 1.00×10^{-1} to 1.00×10^{-5} were made,[16] and 100 µL of each was spread plated onto two media: R2A agar and Tryptic soy agar (Merck, Darmstadt, Germany). All isolation media were prepared with 50.00% Lake Urmia water and supplemented with 25.00 µg mL^{-1} nalidixic acid (PadtanTeb, Tehran, Iran) and 100 µg mL^{-1} cycloheximide (PadtanTeb). No growth occurs in the absence of salt. The strains were incubated at 30.00 ˚C for 5 - 9 days and observed on a daily basis to check the colony growth. Based on morphological features, colonies were picked up and put on modified R2A agar medium with 50.00% sea water and purified using the streak plate method.[17] For long-term storage, pure isolates were frozen at – 80.00 ˚C in Tryptic soy broth medium (Merck) and 20.00 % glycerol.

Characterization of isolated strains and determination of optimal NaCl concentration, pH and temperature. Morphology and physiological studies were performed with cells grown on TSA medium at 30.00 ˚C and pH 7.20. Cell morphology and Gram staining were observed by light microscopy (CX43; Olympus, Tokyo, Japan) in 24-hr cultures.[18] Catalase activity was determined by bubble production in 3.00% (v/v) hydrogen-peroxide solution (Sigma-Aldrich, Taufkirchenm, Germany). Oxidase activity was determined by oxidation of 1.00% p-amino dimethyl aniline oxalate (Sigma-Aldrich). Optical density in the 600 nm was followed as a measurement (Stat Fax 4300 Chromate; Awareness Technology Inc., Florida, USA) for growth temperature, pH and salt requirement with plating isolates in Tryptic soy broth. Temperature optimization was done by culture incubation within the range of 20.00 - 60.00 ˚C at intervals of 10.00 ˚C. For pH tolerance analysis, pH of the medium was determined in the range of 4.00 - 10.00 at intervals of 1.00 pH unit.[19,20] The salt requirement of these strains was determined at different salt concentrations of 0.00, 1.00, 1.50, 3.00, 5.00, 7.50, 10.00, 15.00, 20.00, 25.00 and 30.00% (w/v).[18]

Test organisms. To investigate the antimicrobial potential of the isolates, 29 multi drug resistant strains of *E. coli* were obtained from poultry farm of Urmia, Iran and were transferred via brain heart infusion broth to the laboratory. Aseptic swabs of test strains were cultured on selective differential media eosin methylene blue agar (EMB; Oxoid, Altrincham, UK). Test organisms were identified as *E. coli* by observing their cultural characteristics, morphology by Gram's stain, oxidase test, biochemical reactions using indole, methyl-red, Voges-Preuskuar and citrate tests (IMViC), Kligler Iron Agar (KIA; Merck) and motility test as described by Tonu *et al*.[21] Sensitivity pattern of *E. coli* strains were checked against 10 antibiotics using antibiotic-impregnated disc procured from PadtanTeb. All samples were incubated for 24 hr at 37.00 ˚C in nutrient agar for successful isolation of typical colonies.

Preliminary antibacterial screening of isolated strains. All isolated strains were checked for antibacterial activity against pathogens by cross streak and spot methods. In the cross streak method, single colonies of isolated strains were inoculated as a single middle line at the center of semi-solid (0.80% w/v agar) TSA plate and incubated at 30.00 ˚C for 5 days.[22] The test organisms were streaked perpendicularly to the isolated strains and incubated at 37.00 ˚C for 24 hr. Inhibitions were ranked as: – no inhibition, +/– attenuated growth of test strain, + < 50.00% growth inhibition (less than half of the bacterial line was inhibited), ++ 50.00% growth inhibition (half of the bacterial line was inhibited), +++ > 50.00% growth inhibition (more than half of the bacterial line was inhibited). In spot method, isolates were spot inoculated on TSA plates and incubated at 30.00 ˚C for 5 days. The plates were overlaid with semi-solid (0.60% w/v agar)

Table 1. Information of samples.

Samples	Sampling sites	Location	The characteristic of sample
1	Aq Gonbad	37° 49' 16'' N 45° 24' 19'' E	Sticky sediment
2	Burachalo	37° 56' 24'' N 45° 23' 52'' E	Sticky sediment
3	Kushk	38° 00' 43'' N 45° 21' 13'' E	Sandy soil
4	Sharafkhaneh port	38° 10' 09'' N 45° 26' 08'' E	Black sludge
5	Sheikh Vali	38° 13' 45'' N 45° 22' 59'' E	Sandy soil
6	Qharabagh	38° 11' 57'' N 45° 07' 41'' E	Gray muddy soil
7	Govarchin Ghalea	38° 04' 05'' N 45° 11' 48'' E	Sticky black sludge
8	Bari	38° 00' 13'' N 45° 05' 31'' E	Black sludge
9	Gol Tappeh	37° 54' 33'' N 45° 04' 36'' E	Black sludge
10	Khaneshan	37° 48' 30'' N 45° 14' 26'' E	Sandy soil

nutrient agar medium that were previously seeded with 1.00×10^8 CFU of one of the test microbial strains. The plates were incubated at 37.00 ˚C for 18 - 24 hr and the inhibition zone diameter around the colonies was recorded.[23]

Secondary screening of antibacterial activity. Two isolates that showed antagonistic activity against *E. coli* 14, *E. coli* 244, *E. coli* 180 and *E. coli* 416, were further evaluated using agar well diffusion assay.[24] The selected isolates were grown on Tryptic soy broth at 30.00 ˚C for 9 days. After growth, culture broths were separated by centrifuging at 10,000 rpm for 15 min. The crude supernatant was extracted by vigorously mixing the cell free culture with ethyl acetate (1:1) for 30 min, and the organic fraction was subjected to rotary evaporation under vacuum. The crude bioactive ethyl acetate fraction was tested for secondary screening using Mueller Hinton agar medium (Oxoid) that was previously seeded with 1.00×10^8 CFU of the mentioned pathogens. The activity was evaluated by adding 50.00 µL of the supernatant to solidified agar medium. The zones of inhibition were measured after 24 hr incubation at 37.00 ˚C and all the assays were repeated in three replicates to confirm the antibacterial activity. Uninoculated broth medium added to the wells were taken as control.

Biochemical testing. After antibacterial screening, the positive isolates were selected for biochemical testing including catalase test, indole test, oxidase test, urease test, starch and casein hydrolysis, using different sugars as carbon source and citrate utilization test (Merck). [25]

Antimicrobial susceptibility assay. Antibiotic susceptibility was carried out on Mueller-Hinton agar against selected strains using disc diffusion technique.[26] Following antibiotics were tested: ceftriaxone (30.00 µg), kanamycin (30.00 µg), imipenem (10.00 µg), erythromycin (15.00 µg), tetracycline (30.00 µg), ciprofloxacin (5.00 µg), gentamicin (10.00 µg), and streptomycin (10.00 µg). The susceptibility or resistance was measured by diameter of the zones of inhibition.[27]

Genomic DNA isolation. The genomic DNA of strains was extracted using popularly known boiling method described by Wilson *et al*. with slight modifications. Each isolate was cultivated in TSA for 24 hr prior to the extraction procedures. A single colony of pure cultures was suspended in 400 µL dH_2O and followed by two cycles of heating at 100 ˚C for 10 min. The lysed suspensions were centrifuged for 5 min at 5,000 *g* and supernatant was transferred into a fresh tube. Genomic DNA was stored at – 20.00 ˚C for short term until required.[24]

Molecular identification and phylogenetic analysis of halophilic strains. For 16S rRNA gene amplification, universal primers 27F (5 - AGAGTTTGATCMTGGCTCAG-3) and 1492R (5 - GGTTACCTTGTTACGACTT-3) were used in the PCR reaction.[28] Each 50.00 µL PCR reaction contained 50.00 ng genomic DNA, 0.20 µM each of primers, 0.20 mM of each dNTP (25.00 mmol L^{-1}), 1X SmarTaq buffer (10X), 1.50 mM $MgCl_2$ (50.00 mmol L^{-1}) and 1.00 U SmarTaq DNA polymerase (CinnaGen, Tehran, Iran). The reaction mixtures were placed in a thermocycler (Applied Biosystems, Waltham, USA) using the following reaction conditions: initial denaturation step at 94.00 ˚C for 5 min, followed by 35 cycles at 94.00 ˚C for 30 sec, annealing at 55.00 ˚C for 30 sec, and extension at 72.00 ˚C for 30 sec, with a final extension at 72.00 ˚C for 7 min. The PCR products were evaluated by 1.00% (w/v) agarose gel electrophoresis and products were quantified and submitted for sequencing by ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). The 16S rRNA sequences were subjected to BLAST (The Basic Local Alignment Search Tool) search to obtain similarity between sequences and aligned manually. The phylogenetic tree was constructed by the Maximum Likelihood method, using the program MEGA X version 6 and bootstrap values are given.[29,30]

Minimum inhibitory concentration (MIC). Minimum inhibitory concentration assay was performed according to Singh *et al*. by broth microdilution assay.[23] Different concentrations of the extract (100, 50.00, 25.00, …, 1.56 µg mL^{-1}) of isolates LUH16 and LUH20, were prepared by two-fold serial dilution and were added to pre coated microbial strains in 96-well plates. The plates were incubated for 24 hr at 37.00 ˚C and absorbance of each well was measured at 630 nm using the Chromate 4300 microplate reader. The MIC is defined as the lowest concentration required to inhibit the bacterial growth. Each test was done in triplicate.

Gas chromatography-mass spectrometry analysis. The crude bioactive ethyl acetate fraction was subjected to GC-MS analysis using the protocol of Ser *et al*. with slight modifications.[31] For GC-MS analysis a gas chromatograph (7890A; Agilent Technologies Inc., Santa Clara, USA) coupled to a 5975C mass spectrometer with an HP-5MS capillary column (5.00% phenyl methyl polysiloxane, 30.00 m × 0.25 mm × 0.25 µm) was used. The oven temperature was programmed initially at 80.00 ˚C for 3 min, followed by an 8.00 ˚C per min increase in temperature to 180 ˚C and was kept isothermally for 10 min. Helium gas (1.00 mL per min) was used as the carrier gas to inject 0.20 µm-filtered samples with ionization voltage at 70.00 eV. The injector was set in a split mode (split ratio of 1:500) and mass range acquisition was from 40.00 to 500 m/z. The constituents were identified by comparing the obtained mass spectra with the data available in the NIST 05 spectral library.

Results

Isolation and identification of halophilic strains. Samples were collected from the ten different coastal areas of Lake Urmia (north part). Their dominant distribution according to the sampling site was: 40.00% from Bari coast, 30.00% from Gol Tappeh, 15.00% from

Sharafkhaneh port and 15.00% from Aq Gonbad. In situ Analysis of physical parameters indicated that samples had a neutral pH of 7.30 ± 0.20 and a temperature range of 12.00 - 19.00 ˚C. To assess the bioactive halophilic bacteria, selective isolation was done using TSA and R2A medium supplemented with sea water (50.00% v/v). In this study a total of 20 halophilic strains were isolated from the Lake, in which the most abundant genus was *Halomonas*.

Antimicrobial screening. All isolates were subjected to evaluate the MIC value against different strains of avian *E. coli* using the cross-streak and spot inoculation methods. Totally, 29 resistant *E. coli* were collected from aviculture located in Urmia. The MIC values were measured in mm. In the present study, *E. coli* strains were found to be resistant to the majority of the tested antibiotics, except for cefoxitin, ceftazidime, and nitrofurantoin. Among the investigated antibiotics, tetracycline and erythromycin are widely used in poultry farms. The high resistance to these antibiotics reflects their widespread use in poultry. Among the 20 halophilic isolates, two isolates, LUH16 and LUH20, exhibited selective antimicrobial activity against four *E. coli* strains (*E. coli* 14, *E. coli* 244, *E. coli* 180, and *E. coli* 416). In cross streak method the inhibition patterns of isolates against different strains of *E. coli* were slightly dissimilar with a diameter of inhibition zone ranging from 12.00 to 20.00 mm (Table 2).

Table 2. Antimicrobial activities of *Halomonas* isolates against multidrug resistant avian pathogenic *E. coli*.

Active strains	Inhibition zone diameter (mm)			
	***E. coli* 14**	***E. coli* 244**	***E. coli* 180**	***E. coli* 416**
LUH20	++ (17.00)	++ (16.00)	+ (12.00)	++ (16.00)
LUH16	++ (18.00)	+ (14.00)	++ (16.00)	++ (20.00)

The LUH20 and LUH16 strains that exhibited efficient antimicrobial activities in cross streak method also showed similar activities in spot inoculation assay. In this method maximum antimicrobial activity (> 26.00 mm) was recorded against resistant *E. coli* 14 (Fig. 1). This indicates that the potential for these two strains can lead to the further investigation towards avian pathogenic *E. coli*. To confirm the production of antimicrobial metabolites, the extracts of bioactive bacterial isolates were used for agar well diffusion assay. In this assay a range of inhibitions of *E. coli* 14, *E. coli* 244, *E. coli* 416 and *E. coli* 180 were observed by LUH20 24.60; 22.00; 21.30; 20.00 mm and by LUH16 26.00; 21.00; 23.00; 17.00 mm, respectively. Different concentrations (25.00, 50.00, 75.00, 100 µL) of selected isolates showed different zone of inhibition. In current study we evaluated the activity by adding 50.00 µL of the supernatant. Overall, the highest antimicrobial activity of LUH20 (24.60 ± 1.00 mm) and LUH16 (26.00 ± 1.00 mm) strains was shown against *E. coli* 14 similar to the previous results. These active isolates mostly originated from Bari coast. This indicated that the marine habitats of Lake Urmia harbor unique bioactive bacteria. To study the interaction of LUH20 and LUH16 with test microbial strains, the MIC of the extracts was evaluated. The isolate, LUH20, showed the strongest antimicrobial activity against *E. coli* 14, with MIC values of 31.2 µg mL^{-1}. For other *E. coli* strains, the MIC values varied between 62.50 - 500 µg mL^{-1}. The isolate LUH16, was active against selected test microorganisms. The MIC for *E. coli* 14 was 62.50 µg mL^{-1}, followed by *E. coli* 416 (125 µg mL^{-1}). The *E. coli* 244 and *E. coli* 180 had the same MIC value at 250 µg mL^{-1}.

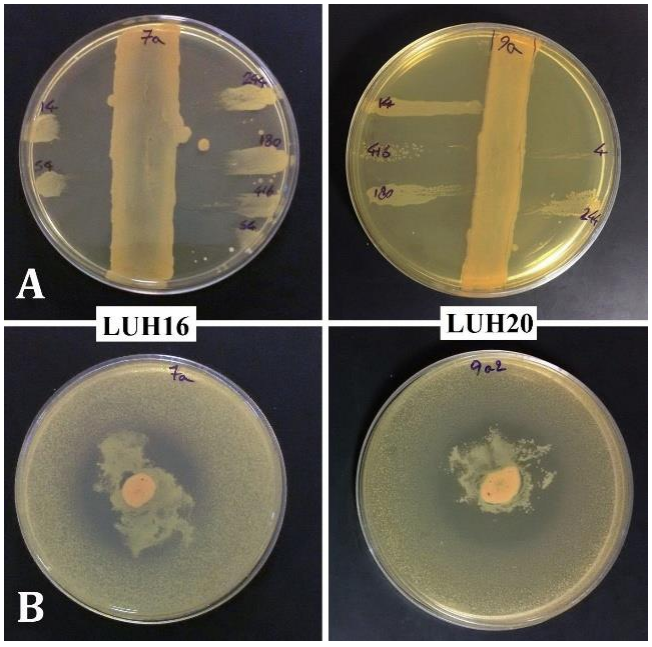

Fig. 1. Screening of antibacterial activity of *Halomonas* sp. against antibiotic-resistant *E. coli* isolates. Screening was done by **A)** cross-streak and **B)** spot inoculation method.

Characterization of the strains LUH20 and LUH16. The isolated strains were analyzed for physiological and biochemical characteristics (Table 3). The strains LUH20 and LUH16 were aerobic, Gram-negative rods, non-motile and non-sporulating bacteria. Colonies on TSA and R2A media were small, cream, smooth and 0.50 - 1.00 mm in diameter after 48hr growth at 30.00 ˚C. The strains were moderately halophilic, growing in a wide range (1.00 - 10.00 %, w/v) of salt concentrations, with optimum growth at 5.00 - 7.50% (w/v) salts. No growth was observed in the absence of NaCl. Both Strains were able to grow over a temperature range of 20.00 - 50.00 ˚C with optimum growth at 30.00 ˚C and pH 6.00 - 9.00 with optimum growth in alkaline pH 8.00. The two selected bioactive isolates were subjected to various biochemical tests. In this study, both *Halomonas* strains were negative for indole production. Biochemical characters suggested that the LUH20 strain utilizes galactose, glucose, lactose, and sucrose as sources of carbon, whereas sucrose is not

utilized by the LUH16 strain. Both strains were positive for urease, hydrolysis of starch, and casein; however, the oxidase test showed negative results for both strains. Additionally, only the LUH20 strain tested positive for the hydrolysis of Tween 80, while the LUH16 strain did not show hydrolytic activity. As shown in Table 3, the antibiogram assay indicated that two *Halomonas* strains were susceptible to all of the tested antibiotics except for tetracycline and erythromycin that showed resistance.

Table 3. Physiological and Biochemical characteristics of isolate LUH20 and LUH16 of the genus *Halomonas.*

Characteristic	LUH20	LUH16
Morphology	Long Rods	Short Rods
Colony pigmentation	Cream	Cream
NaCl range (%, w/v)	1.00 - 10.00	1.50 - 10.00
NaCl optimum (%, w/v)	3.00 - 5.00	5.00
Temperature range (°C)	20.00 - 50.00	20.00 - 50.00
Temperature optimum (°C)	30.00	30.00
pH range	6.00 - 9.00	6.00 - 9.00
Oxidase	–	–
Indole	–	–
Citrate (Simmons)	+	+
Catalase	+	–
Urease	+	+
Hydrolysis of:		
Casein	+	+
Starch	+	+
Tween 80	+	–
***Carbon utilization*:**		
Glucose	+	+
Galactose	+	+
Lactose	+	+
Sucrose	+	–
Sensitivity		
Ciprofloxacin	+	+
Ceftriaxone	+	+
Gentamicin	+	+
Kanamycin	+	+
Imipenem	+	+
Streptomycin	+	+
Erythromycin	–	–
Tetracycline	–	–

Phylogenetic analysis. Taxonomic status by sequencing of partial 16S rRNA gene (approximately 1500 bp; Fig. 2) and BLAST search in the NCBI revealed that these two strains belong to the genus *Halomonas*. The 16*S* rRNA gene sequences of these isolates *Halomonas* sp. strain LUH16 and *Halomonas* sp. strain LUH20 are available on the GenBank® nucleotide database under the accession numbers MN428653 and MN428654, respectively. Despite the limited research about the antibacterial potential of this genus, our result confirmed that *Halomonas* species appeared to be good antibiotic candidates against Multi drug resistant strains of *E. coli*. The phylogenetic relationship of these strains is very close to several strains of the *Halomonas* genus, so that more similar sequences are in the same cluster. Due to this high similarity, the phylogenetic tree was not drawn. The genotypic and phylogenetic characteristics of these bioactive strains are very closely related to each other and to *Halomonas salifodinae* BC7 (100% identity between the 16S rRNA gene sequences). We have found some differences with respect to the physiological and biochemical responses (oxidase, catalase, Tween hydrolysis, sucrose utilization) of these strains.[32]

Gas chromatography-mass spectrometry analysis. Identification of the bioactive constituents was carried out by GC-MS analysis of the crude ethyl acetate extract of LUH16 and LUH20 strains (Fig. 3). The GC-MS analysis showed the presence of eight bioactive compounds in the samples on comparison of the mass spectra, molecular weight, retention time, and molecular formula with the NIST library. The identified compounds are listed in Table 4. The bioactive compounds identified in the ethyl acetate extract of the LUH16 strain were as "Bis (2-ethylhexyl) phthalate", "7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione", "Octadecanoic acid, ethyl ester", and "Heptadecanoic acid, ethyl ester". The major metabolites present in LUH20 strain were "Oxime-, methoxy-phenyl" and "Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)". Other compounds "viz. Benzoic acid, 3,5-dihydroxy" and "Quinoline, 2-ethyl" were also detected in both strains with different retention times. The structures of the bioactive constituents are given in Figure 4.

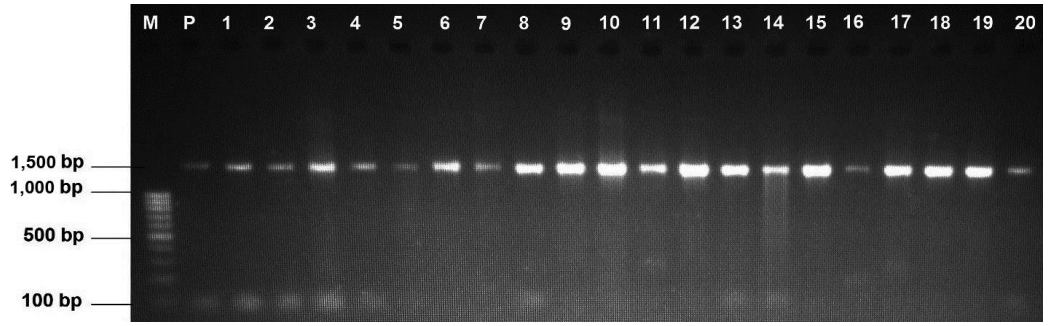

Fig. 2. Polymerase chain reaction products from 16S rRNA gene amplification using 27F and 1492R primers on agarose gel. M: Marker (100 bp Ladder); P: positive control; 1-20: Halophilic bacteria isolated from Lake Urmia.

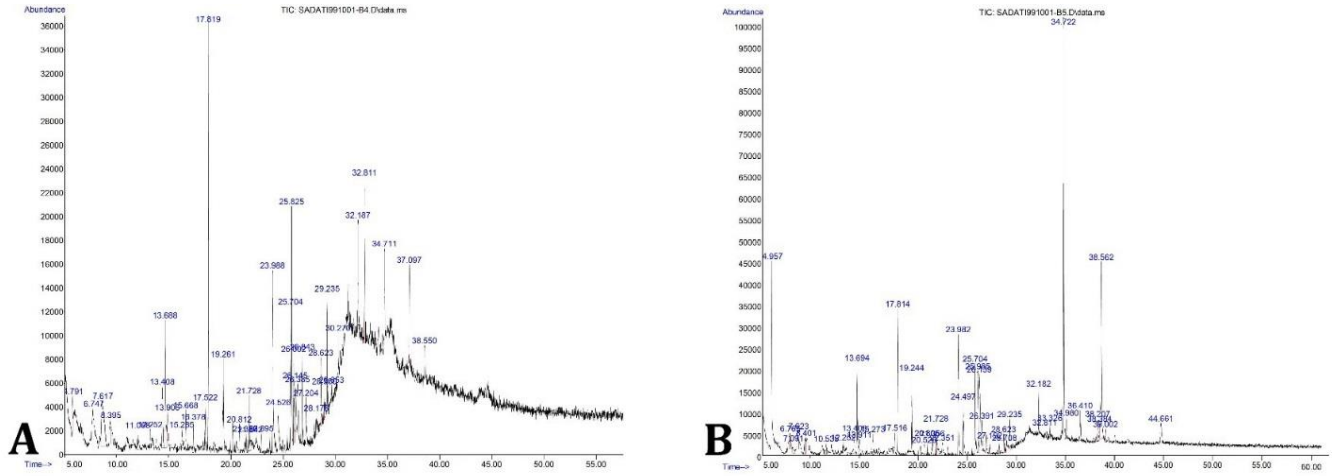

Fig. 3. The GC-MS chromatogram of the crude compounds from *Halomonas* sp. LUH16 (a) and *Halomonas* sp. LUH20 (b).

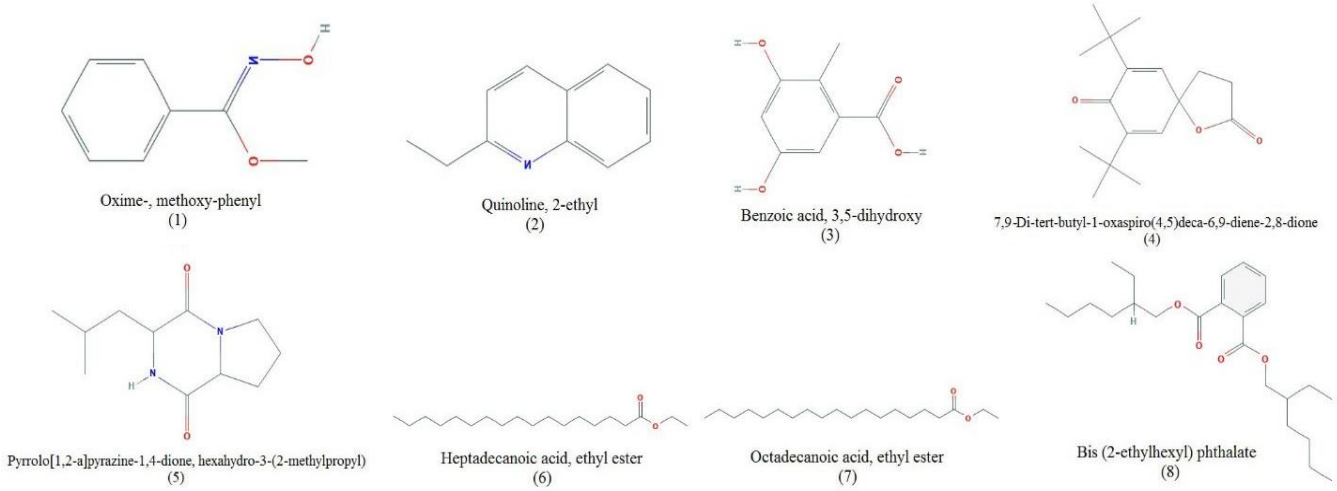

Fig. 4. Chemical structures of the identified compounds from *Halomonas* sp. LUH16 and *Halomonas* sp. LUH20 (https://www.ncbi.nlm.nih.gov/pccompound).

Table 4. Compound identified from *Halomonas* sp. LUH16 and *Halomonas* sp. LUH20 extracts using GC-MS.

Sample	RT (min)	Compound name	Formula	Molecular weight (g mol)	Area (%)	Activity	References
1	4.96	Oxime-, methoxy-phenyl	$C_8H_9NO_2$	151	7.45	Antioxidant and Antimicrobial	33
2	19.26	Quinoline, 2-ethyl	$C_{11}H_{11}N$	157	4.15	Antibacterial, antifungal, antiviral, and anti-parasitic	41
3	25.70	Benzoic acid, 3,5-dihydroxy	$C_7H_6O_4$	154	3.51	Antimicrobial	37
4	25.83	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	4.46	Antimicrobial	36
5	25.99	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	210	3.34	Antimicrobial and Antioxidant	39
6	26.84	Heptadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	270	1.91	Antimicrobial	40
7	29.24	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	2.47	Antioxidant, anti-inflammatory	38
8	32.81	Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	5.28	Antimicrobial and Cytotoxic	35

RT: Retention time.

Discussion

As the result of the article shows, the high frequency of antimicrobial resistance has made *E. coli* a potential problem in poultry industry, due to the possibility of horizontal transfer of antimicrobial resistance to the human resident microbiota. Replacing bioactive compounds of our isolates with synthetic antibiotics in poultry production may be contributing to the lower frequency of bacterial resistance to antimicrobials, leading to a lower risk of their transmission to humans.

In our study, Oxime-methoxy-phenyl and Bis (2-ethylhexyl) phthalate were identified as dominant metabolites. Oximes are naturally occurring compounds and are also obtained by chemical synthesis. Oximes are known to exhibit a wide range of bioactivities including anti-inflammatory, antimicrobial, antioxidant, and anticancer activities.[33] In this study, the oxime- methoxy-phenyl compound with 90.00% probability in LUH20 extract showed a broad-spectrum of activity, particularly against *E. coli* 14. Recently, this bioactive compound has also been obtained from *Streptomyces pratensis* QUBC97.[34] The major compound from LUH16 strain included Bis (2-ethylhexyl) phthalate, which is known for antimicrobial and cytotoxic activities. Although, several studies have introduced Actinomycetes and *Bacillus cereus* as the predominant producers of this compound mainly against Gram-negative bacteria,[35] there is no much literature reporting the production of Bis (2-ethylhexyl) phthalate by other genera of bacteria. Another compound present in LUH16 isolate in good amount was 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, which was characterized as a phytochemical metabolite having antimicrobial activities against *E. coli*, *Staphylococcus aureus*, *Salmonella Typhi*, and *Pseudomonas aeruginosa*.[36]

The present study showed that Lake Urmia ecosystem harbors potential halophilic bacteria, especially *Halomonas*, capable to produce bioactive metabolites that are specifically antimicrobial towards antibiotic-resistant strains of *E. coli*. Out of 20 isolates obtained, only two isolates could be considered as effective antimicrobial metabolites producers. Most compounds acquired from our isolates such as Bis (2-ethylhexyl) phthalate, a very common plastic softener can be found in nearly all extracts from natural sources and there are many reports describing their biological activity. However, the biological activity of the compounds is seen in mixture with phthalate and have no effect alone.

These findings makes *Halomonas* strains a future candidate for drug prospecting studies. To our knowledge, these findings can be the first report of *Halomonas* strains displaying antimicrobial activity against avian *Escherichia*.

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

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