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ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL METABOLITES PRODUCING BACTERIA FROM SOILS IN AL-AHSA, SAUDI ARABIA

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ABSTRACT

The current study aims to isolate and test microorganisms from soils in Al-Ahsa, Saudi Arabia, for their antibiotic-producing ability. A total of fourteen bacterial isolates were obtained from six locations, including poultry house soil, Al-Asfar Lake soil, Nada dairy products farm soil, the rhizosphere of *Prosopis farcta* in Ayen Al-Harrah, Circular Road desert soil, and Al-Uqair beach soil. Screening revealed antibacterial activity in six of the fourteen isolates against specific pathogens. Notably, *Salmonella enterica* was inhibited by isolates NP1 and NP2. Additionally, NY1 inhibited NY2 and NP2, NN1 inhibited NN2, NH1 inhibited NH2, and NC1 inhibited NC2. Morphological and biochemical differences were observed among the isolates. The disc diffusion method indicated a minimum inhibitory concentration (MIC) range of 0.78 – 12.5 µg/µl for the antibiotic-producing isolates. Gas chromatography- mass spectrometry (GC-MS) analysis of these isolates revealed several secondary metabolites from various chemical classes, including cyclohexasiloxane, dodecamethyl-, cycloheptasiloxane, tetracadmethyl, and phenol 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-], which may inhibit harmful microorganisms. Phylogenetic analysis based on 16S ribosomal ribonucleic acid (16S rRNA) gene sequencing showed that most isolates belonged to the genera *Bacillus* and *Alcaligenes*. NP1 exhibited 98.76% sequence similarity to *Bacillus subtilis* strain HAU427, while NP2 showed 99.65% homology with *Bacillus amyloliquefaciens*. Isolates NY1, NN1, and NC2 were closely related to *Bacillus thuringiensis*, whereas NY2, NN2, and NH2 resembled *Bacillus pumilus*. NH1 was similar to *Bacillus cereus* (97.41%), and NC1 closely matched *Alcaligenes* sp.. The ability of these soil-derived bacterial isolates to inhibit multiple bacterial strains at low concentrations suggests their potential as a source of antibacterial agents against antibiotic-resistant pathogens.

Keywords: Antibiotics, Al-Ahsa, Bacteria, Soil, Minimum Inhibitory Concentration (MIC), GC-MS

INTRODUCTION

Antibiotics, typically derived from microorganisms, kill or suppress microbial pathogens **(Veerapagu *et al.*, 2022)**. During the early stages of antibiotic discovery, compounds such as penicillin (from fungi) and streptomycin and tetracycline (from bacteria) were identified (**Caulier *et al.*, 2018)**. Advances in science now enable laboratories to produce large quantities of antibiotics under controlled conditions, optimizing factors like medium temperature and pH to maximize yield **(Alamri, 2015)**. Soil serves as a reservoir of diverse microbial populations **(Ananbeh *et al*., 2020)**. The abundance, activity, and diversity of soil microbes are influenced by various biotic and abiotic factors, including (1) ambient conditions provided by plant species, (2) soil characteristics, (3) biochemical properties, (4) nutrient levels, (5) acidity, and (6) water content **(Mushtaq *et al*., 2023)**. Most bioactive microbes reside in the top layers of cultivated fields, continental crust rocks, hot springs, and deep ocean sediments **(Prashanthi *et al*., 2021)**. Many soil microbes, including bacteria, produce antibiotics as secondary metabolites to combat various microbial infections **(Prashanthi *et al*., 2021)**. Traditionally, researchers identified antibiotic-producing bacteria using phenotypic features, a method that limited the number of species found. However, techniques such as polymerase chain reaction (PCR) and next-generation sequencing have revolutionized this field, leading to the discovery of numerous novel antibiotic-producing bacteria in soil. Continuous research and the development of new antibiotics are crucial to addressing multi-drug-resistant (MDR) organisms, including MRSA, vancomycin-resistant cocci, *Salmonella*, and *Klebsiella pneumoniae* **(Church, 2022)**. Actinobacteria, particularly the genus *Streptomyces*, are well-known antibiotic producers. *Streptomyces* generates bioactive compounds used in culinary, chemical, and pharmaceutical applications. Similarly, the genus *Bacillus* also produces antibiotics **(Alam et *al*., 2022)**. Gram-positive, endospore-forming *Bacillus* strains can thrive in various environments due to their broad physiological capabilities **(Cawoy *et al*., 2015)**. *Bacillus subtilis*, a common model organism for bacterial research, is recognized for its enzyme production and potential to synthesize numerous antibiotics **(Yunus *et al*., 2016)**. Another notable species, *Bacillus pumilus*, is known for producing a range of antibacterial agents, with *B. pumilus* SAFR-032 identified as a significant antibiotic producer in Saudi Arabia **(Qureshi and Elhassan, 2016)**. This strain produces an 8,000-Dalton peptide that exhibits enhanced activity at acidic pH levels **(Qureshi and Elhassan, 2016)**. Other antibiotic-producing *Bacillus* species found in the region include *B. polymyxa*, *B. licheniformis*, *B. cereus*, and *B. mycoides* **(Al-Humam, 2016)**. The current study investigates antimicrobial metabolites producing bacteria from Al-Ahsa soil in Saudi Arabia. The specific objectives include isolating and screening Al-Ahsa soil bacteria for antibacterial bioactivity, selecting isolates with the most effective antimicrobial activity against pathogens, and performing morphological characterization. Additionally, the study aims to extract secondary metabolites, assess the MIC of bacterial extracts, and determine secondary metabolites using GC-MS analysis. The 16S rRNA gene will be utilized to identify the bacterial isolates exhibiting the highest antimicrobial activity.

MATERIAL AND METHODS

Sample collection and bacterial isolation

Six soil samples (5 g) were collected in sterilized plastic bags from in Al-Ahsa region, Saudi Arabia, in November 2022 (Table1).

Table 1 Soil samples sites and their location maps

Sr. No.	soil samples	Location maps
1	Poultry House soil	25°28'57.5"N 49°33'37.5" E
2	AL-Asfar Lake soil	25°23'24.2"N 49°37'11.8" E
3	Nada dairy products farm soil	25°32'16.7"N 49°37'00.9" E
4	The rhizosphere of P. farcta in Ayen Al-Harrah	25°23'24.2"N 49°37'11.8" E
5	circular road desert soil	25°17'59.6"N 49°33'42.3" E
6	Al-Uqair beach soil	25°47'05.4"N 50°10'07.0" E

Bacterial isolation was carried out using the pour plate method as described by **Hussein *et al*. (2022)**. Exactly 0.2 g of each soil sample was diluted in 40 ml of 0.85% NaCl solution under aseptic conditions. Then, 25µl of each diluted sample was dispensed into an empty petri dish, which was then flooded with nutrient agar (NA) medium (M561A-500G, Himedia, India). The chemical composition of NA medium in one litter of distilled water was presented in table 2 **(Saha and Paul,**

2014) (Table 2). Inoculated plates were incubated at 28 °C for 1-2 days. After the incubation period, colonies with distinct morphology and colonies that have antagonistic activity against each other were re-streaked onto fresh NA plates using sterile loop, to ensure the purity of the isolates. Inoculation plates were incubated at 28°C for 24 - 48 h.

Table 2 Chemical composition of NA medium

Ingredients	Concentration (g\ l)
Peptone	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000

Primary screening of antibiotic-producing bacteria

Antimicrobial Activity of Soil Isolates against Pathogenic Microbes

The antimicrobial activity of soil isolates against nine pathogens, including Gram-negative bacteria and Gram-positive bacteria, in addition to one fungal strain, *Candida albicans* ATCC10231 (Table 3) was tested according to the previously mentioned method of **Bala *et al.* (2012)** with the following modification. Briefly, one colony from each of the pathogens was suspended with 10 ml of 0.85% NaCl and mixed well using a shaker incubator for 10 min. Then, 25 µl from the suspension was spread homogeneously onto NA plates. One colony of each of the soil isolates was carefully placed onto inoculated plates followed by incubation at 28°C for 24-48h. After incubation, plates were checked visually for the formation of inhibition zones around soil-tested isolates.

Table 3 Strains code of pathogenic strains according to Clinical Microbiology Procedures Handbook.

Pathogenic bacterial isolate	Strain code
Candida albicans	ATCC 10231
Enterococcus faecalis	ATCC29212
E. coli	ATCC 25922
Klebsiella ozaenae	Clinical sample
P. aeruginosa	ATCC 27853
S. enterica	Clinical sample
S. aureus	ATCC 25923
Staphylococcus haemolyticus	Clinical sample
Streptococcus penumomniae	ATCC4961

Antimicrobial Activity of Soil Isolates against each other

The antagonistic activity of bacterial isolates against each other was determined. With the same type of suspension as before, one colony of each of the soil isolates was put on NA plates that had already been inoculated with 25 µl of each of the other isolates. After incubation of inoculated plates at 30 °C for 48 h, plates were checked visually for the formation of inhibition zones around tested isolates. This experiment was performed in triplicate.

Bacterial isolate phenotype

Soil bacterial isolates that produced antibiotics and were sensitive in primary screening were phenotypically characterized. A previously reported approach was used to characterize the selected isolates' colony structure, color, elevation, margin, and Gram staining reaction **(Claus, 1992)**. Biochemical characterization examined the isolates' ability to metabolize the API-20E test kit's 20 substances per manufacturer's directions. Incubated at 28°C, the strips were scored after 24–48h **(Mohammad *et al*., 2020)**.We tested the previously mentioned bacterial isolates for catalase production. A loop of growing isolate was placed on a new glass slide. Some drips of 5% hydrogen peroxide were on the slides. Positive outcomes were seen with gas bubbles **(Bala *et al*., 2012)**.

Find MIC

Extracting Bacterial Secondary Metabolites

One colony of each antibiotic-producing isolate was grown in 50 ml NB medium. Cultures were incubated at 28 °C for 24 hours. Two milliliters of each pre-culture were put to 100 ml of fresh NB medium in a flask (Table 4). The chemical composition of NB media (M002-100G, Himedia, India) in one litter of distilled water was shown in table 4 **(Saha and Paul, 2014)**. A 180-rpm shaking incubator incubated the flasks at 28 °C for 24 hours. After incubation, 200 ml methanol was added to each culture. The organic phase was separated from the sediment by centrifuging the cultures for 15 minutes at 6000 rpm. Finally, a 40 °C hot air oven evaporated the organic phase. The crude extract was formed from secondary metabolites that remained in the beaker after evaporation **(Nxumalo *et al*., 2020)**.

Table 4 Chemical composition of NB medium

Ingredients	Concentration (g\ l)
Peptone	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500

Disc diffusion method (DDM)

The procedure in study of **Keskın *et al*. (2012)** was modified for this experiment. In summary, 50 mg of each solid crude extract was dissolved in 1 ml methanol to create a 50 µg/µl stock solution. This solution was then serially diluted to concentrations of 25 µg/µl, 12.5 µg/µl, 6.25 µg/µl, 3.13 µg/µl, 1.56 µg/µl, and 0.78 µg/µl. Sterile 6 mm filter paper discs were impregnated with 20 µl of each crude extract stock solution. After that, discs were placed on bacterial-inoculated agar. Klavox 1g (Amoxicillin 875mg + Clavulanic acid 125mg) was used as a positive control at 0.016 µg/µl. Distilled water was the negative control. After 24h of incubation at 28°C, the inhibition zone around each disc was measured in mm to calculate MIC, the lowest concentration that prevented bacteria growth **(Nxumalo *et al*., 2020)**.

GC-MS analysis

Sample Prep/Extraction

One colony from each of the six bacterial isolates was suspended in 1 mL of HPLC-grade methanol (96–100%) and kept overnight at 4°C. Clarified supernatants were obtained by filtering samples through 0.22 µm Millipore filters in glass test tubes.

GC-MS analysis

A Perkin-Elmer GC Clarus 500 system was used for GC-MS analysis, featuring an AOC-20i auto-sampler, Gas Chromatograph, and DB-5 capillary column (30 m 0.25 mm ID 0.25 m df, Agilent, Santa Clara, CA, USA) for separation. Helium was used as a carrier gas at a constant flow rate of 3 ml/min, with a 2µl injection volume of crystal-clear sample. Temperature was programmed from 110°C for 2 min, then 10°C/min to 200°C, then 5°C/min to 280°C for 9 min **(Anantha *et al*., 2016)**.

Processing data

We compared the mass spectrum and retention time of unknown bacterial secondary metabolites to those of known components in the NIST database **(Anantha *et al*., 2016)**.

Genotypic characterization of soil bacterial isolates

Molecular Identification Using 16S rRNA Gene Sequencing

The probiotic bacteria were identified using 16S rRNA gene sequencing. From the selected isolates, a commercial DNA purification kit (Qiagen, Madison, WI, USA) retrieved total genomic DNA. The universal primers 27 forward 5/- AGA GTT TGA TCM TGG CTC AG -3/ and 1492 reverse 5/- CGG TTA CCT TGT TAC GAC TT -3/ were used to amplify the 16S rRNA gene using PCR. PCR master mix (Amplicon, Odense, Denmark) and 25 ng of extracted DNA template were added to the process. Quality control used electrophoresis on a 1% agarose gel to see amplified PCR results. Following the manufacturer's instructions, a QI-AGEN kit purified amplicons of the expected size (Qiagen, Madison, WI, USA).

Phylogeny

The sequences were compared to GenBank using BLAST (National Centre for Biotechnology Information, Maryland, USA). The MEGA7 version 11 software was used to generate phylogenetic trees using the neighbor-joining technique **(Tamura and Nei, 1993)**.

Statistics

The statistical studies were done in GraphPad Prism 8.0. One-way ANOVA was used to compare group findings. The significance level was set at $p < 0.05$ to identify significant differences.

RESULTS

Sample collection and bacterial isolation

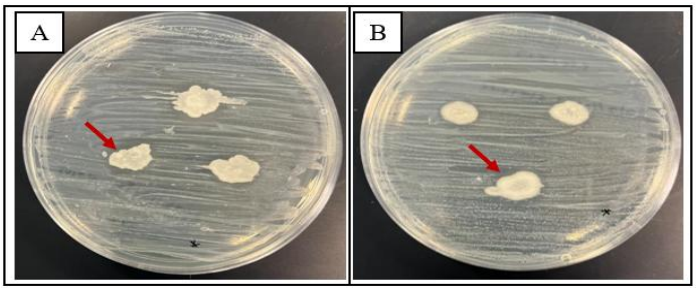
Fourteen bacterial isolates were obtained from soil samples: 2 from a poultry house, 3 from AL-Asfar Lake, 4 from Nada dairy products farm, 2 from the rhizosphere of P. farcta in Ayen Al-Harrah, 2 from Circular Road desert, and 1 from Al-Uqair beach. The isolates from these places were labelled (NP1, NP2),

(NY1, NY2, NY3), (NN1, NN2, NN3, NN4), (NH1, NH2), (NC1, NC2), and (NU1).

Primary antibiotic-producing bacteria screening

Antimicrobial Activity of soil Isolates Against Pathogens

Unlike the other pathogenic isolates, *S. enterica* was responsive to soil isolate growth suppression. NP1 and NP2 inhibited *S. enterica* growth (Figure 1). Table 5



Gave zone of inhibition in mm as from figure1.

Figure 1 Antimicrobial activities of NP1 (A) and NP2 (B) isolates against *S. enterica* after 24h. The arrow indicates the zone of inhibition in each sample. *The growth suppression is nonsignificant.

Table 5 Zones Inhibition (mm) for the NP1 (A) and NP2 (B) isolates against *S. enterica* after 24h

Isolate	Inhibition Zone (mm)
NP1 against *S. enterica* (A)	12
NP2 against *S. enterica* (B)	14

Antimicrobial Activity of soil Isolates Against each other

NY1 suppressed NP2 and NY2 growth. NN1 stunted NN2. NH1 reduced NH2 development and NC1 inhibited NC2 (Figure 2). Table 6 Gave zone of inhibition in mm as from figure2.

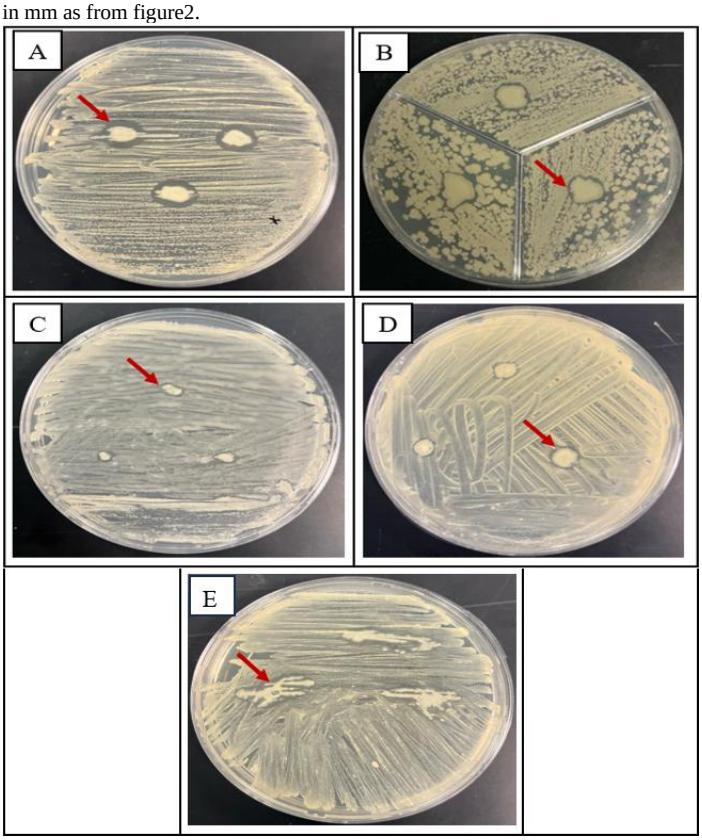


Figure 2 Antimicrobial activities of soil bacterial isolates against each other. As shown NY1 inhibited the growth of NY2 (A) and NP2 (B), NN1 inhibited the growth of NN2 (C), NH1 inhibited the growth of NH2(D), and NC1 inhibited the growth of NC2(E) respectively. The arrow indicates the zone of inhibition in each sample. *The growth suppression is nonsignificant.

Table 6 Zones Inhibition (mm) for the NY1 against NY2 (A), NY1 against NP2 (B), NN1 against NN2(C), NH1 against NH2(D), and NC1 against NC2(E) after 24h.

Isolate	Inhibition Zone (mm)
NY1 against NY2 (A)	10
NY1 against NP2 (B)	9
NN1 against NN2 (C)	5
NH1 against NH2 (D)	8
NC1 against NC2 (E)	9

Phenotypic characterization of bacterial isolates

Depending on primary screening, ten bacterial isolates were Phenotypically characterized according to their potential for antibiotic production like NP1, NP2, NY1, NN1, NH1, and NC1, and their sensitivity to the antibiotics that were produced like NY2, NN2, NH2, and NC2.

Morphological characterization

All ten bacterial isolates were characterized morphologically based on the shape, color, edges, elevation, and gram reaction of their colonies as shown in table 7.

Table 7 Morphological characteristics of bacterial colonies in NA medium

Strain	Shape	Color	Margin	Elevation	Gram reaction
NP1	Circular	Creamy white	Undulate	Flat	Gram-positive
NP2	Circular	White	Undulate	Raised	Gram-positive
NY1	Circular	Creamy white	Entire	Raised	Gram-positive
NY2	Circular	White	Entire	Flat	Gram-positive
NN1	Circular	Creamy white	Entire	Raised	Gram-positive
NN2	Circular	White	Entire	Raised	Gram-positive
NH1	Circular	Off-white	Entire	Flat	Gram-positive
NH2	Circular	White	Entire	Flat	Gram-positive
NC1	Circular	White	Entire	convex	Gram-negative
NC2	Circular	White	Entire	Raised	Gram-positive

Biochemical characterization

API 20E Kit

Isolates were characterized using the API 20E biochemical identification method, which has 20 assays. The results are in Table 3. All bacterial isolates except NY2, NN2, and NH2 were unable to use ONPG. Only isolate NN2 had urease and alcohol dehydrogenase (ADH) activity, which reduced acetaldehyde to ethanol in the last step of alcoholic fermentation. Except for NP1, NP2, and NC2, all isolates used citrate (CIT) for carbon and energy. Isolate NC1 produced indole (IND) indicating tryptophanase hydrolysis. Besides NY2 and NH2, all isolates showed gelatin hydrolysis (Table 8). All bacterial isolates gave negative results for lysine decarboxylation (LDC), hydrogen sulfide production (H_2S), ornithine decarboxylation (ODC), rhamnose fermentation (RHA), melibiose fermentation (MEL), and arabinose fermentation (ARA) (Table 6).

Table 8 Characteristics of strains using API20E

Test	Positive Result
Ortho-nitrophenyl- β-galactoside (ONPG)	NY2 – NN2 – NH2
Citrate utilization (CIT)	All Positive except NP1 – NP2 – NC2
Urease Production (URA)	NN2
Arginine dihydrolase (ADH)	NN2
Indole production (IND)	NC1
Gelatinase production (GEL)	All Positive except NY2 – NH2
Glucose fermentation (GLU)	All Positive except NP2
Mannose fermentation (MAN)	All Positive except NP2
Inositol fermentation (INO)	All Positive except NP2 – NY2 – NH2
Sorbitol fermentation (SOR)	All Positive except NP2 – NY2 – NH2
Amygdalin fermentation (AMY)	All Positive except NP2
Sucrose fermentation (SAC)	All Positive except NP2

Catalase test

Excepting NP1, the remaining 9 bacterial isolates were catalase positive, as indicated by immediate effervescence and formation of gas bubbles upon the addition of hydrogen peroxide to the growing cultures.

Secondary Metabolite Extraction and minimum inhibitory concentration (MIC) determination

Disc Diffusion Method (DDM)

Disc diffusion was used to evaluate crude extracts from isolated microorganisms for antibiotic activity. MIC values varied by extract and test strain. Each crude extract had antimicrobial properties. The positive control, Klavox, worked against all strains. The NP1 crude extract inhibited *S. enterica* growth at a MIC of 3.13 µg/µl (Table 9). The NP2 crude extract inhibited *S. enterica* with a lower MIC value of 1.6 µg/µl (Table 9). The NY1 crude extract had MIC values of 6.25 µg/µl for isolate NY2 and 0.78 µg/µl for isolate NP2 (Table 9). The NN1 crude extract inhibited NN2 isolate with a MIC value of 1.56 µg/µl (Table 9). The crude NH1 extract inhibited NH2 isolate with a MIC value of 12.5 µg/µl (Table 9). The NC1 crude extract inhibited NC2 isolate with a MIC value of 1.56 µg/ µl (Table 9).

Table 9 Zone Inhibition (mm) of extracts of various bacterial strains

Extract concentration (µg/µl)	Zone of inhibition (mm)					
	NP2	NY2	NP2	NN2	NH2	NC2
50	17± 1.2	14± 0.1.5	9± 0.9	13± 2.1	11± 1.4	16± 2.8
25	16± 1.0	12± 1.1	8± 0.85	10± 1.4	10± 1.09	15± 2.1
12.5	15± 0.9	10 ± 1.4	7± 0.87	8± 1.1	7± 0.96	13± 1.4
6.25	14± 0.8	9± 1.1	6± 0.63	7± 0.93	0	10± 1.12
3.13	12± 0.9	0	5± 0.57	6± 0.75	0	9± 0.85
1.56	8± 0.8	0	4± 0.44	5± 0.42	0	8± 0.77
0.78	6± 0.66	0	3.2±0.28	0	0	0

GC-MS analysis

The methanol extract of the antibiotic-producing samples, NP1, NP2, NY1, NN1, NH1, and NC1, yielded a total of bioactive components identified by the GC-MS analysis. Tables (10–15) and Figures (3-8) below represent the main compounds obtained from each sample extract, according to area%.

Table 10 Dominant components identified in NP1 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	Cyclohexasiloxane, dodecamethyl-	8.862	24.81
2	Cycloheptasiloxane, tetradecamethyl-	11.08	17

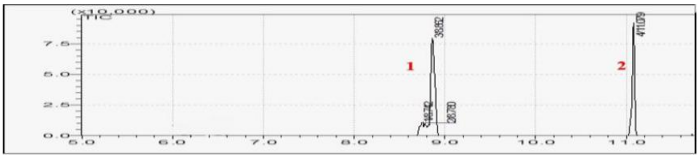


Figure 3 Dominant components identified in NP1 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for Cyclohexasiloxane, dodecamethyl-. Peak 2 for Cycloheptasiloxane, tetradecamethyl-.

Table 11 Dominant components identified in NP2 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	Cyclohexasiloxane, dodecamethyl-	8.822	23.18
2	Cyclopentasiloxane, decamethyl-	6.348	19.66
3	Cycloheptasiloxane, tetradecamethyl-	11.061	14.04

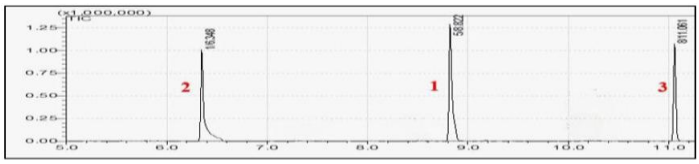


Figure 4 Dominant components identified in NP2 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for Cyclohexasiloxane, dodecamethyl-. Peak 2 for Cyclopentasiloxane, decamethyl-. Peak 3 for Cycloheptasiloxane, tetradecamethyl-.

Table 12 Dominant components identified in NY1 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	Cyclopentasiloxane, decamethyl-	6.372	15.22
2	Docosanoic acid, ethyl ester	17.009	13.04
3	Cyclohexasiloxane, dodecamethyl-	8.842	11.27

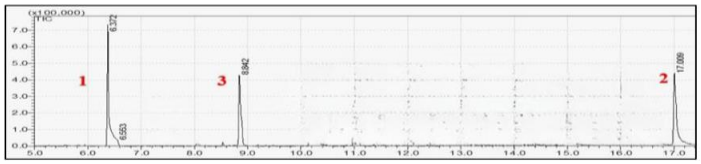


Figure 5 Dominant components identified in NY1 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for Cyclopentasiloxane, decamethyl-. Peak 2 for Docosanoic acid, ethyl ester. Peak 3 for Cyclohexasiloxane, dodecamethyl-.

Table 13 Dominant components identified in NN1 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	Cyclohexasiloxane, dodecamethyl-	8.837	14.23
2	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	20.783	11.56
3	Cycloheptasiloxane, tetradecamethyl-	11.072	10.27

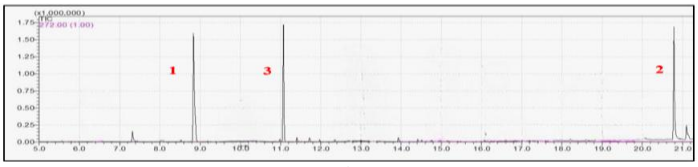


Figure 6 Dominant components identified in NN1 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for Cyclohexasiloxane, dodecamethyl-. Peak 2 for Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-. Peak 3 for Cycloheptasiloxane, tetradecamethyl-.

Table 14 Dominant components identified in NH1 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	20.787	11.77
2	Cyclohexasiloxane, dodecamethyl-	8.841	10.96
3	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	23.476	10.12

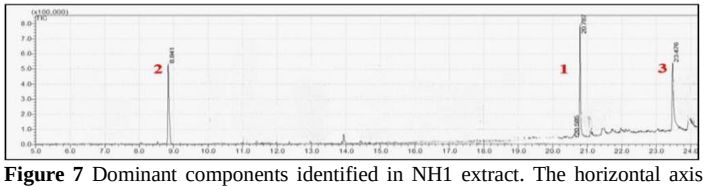


Figure 7 Dominant components identified in NH1 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl. Peak 2 for Cyclohexasiloxane, dodecamethyl-. Peak 3 for 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester.

Table 15 Dominant components identified in NC1 extract with their area% and retention time (Rt)

Number	Compounds	Rt	Area%
1	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	23.476	25.84
2	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	20.786	19.48

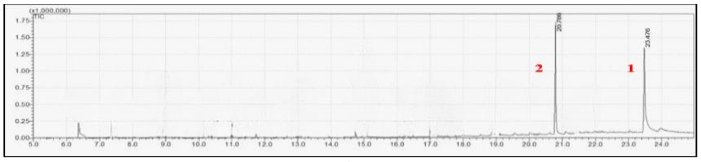


Figure 8 Dominant components identified in NC1 extract. The horizontal axis represents the retention time (Rt), the major axis represents the detector, and the peaks represent the concentration of components in the sample. Peak 1 for 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester. Peak 2 for Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-.

Genotypic characterization of soil bacterial isolates

Molecular Identification Using 16S rRNA Gene Sequencing

The 16S ribosomal RNA gene was amplified from ten bacterial isolates using PCR with universal primers 27F/1492R. The size of the PCR product was nearly 1500 bp as indicated by DNA ladder as shown in figure 9.

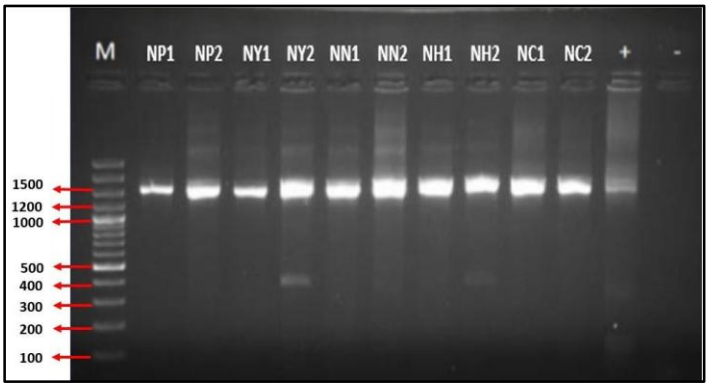


Figure 9 Agarose gel electrophoresis of PCR amplified products of ten tested samples. Lane M, molecular weight marker; Lanes from 2 to 11 positive bacterial 16S rRNA PCR products. Lane 12 confirmed positive *Mycobacterium avium*. Paratuberculosis DNA extract served as positive control and Lane 13 negative control, Nuclease free water served as negative control.

Table 16 Identity percentages of the bacterial isolates to the closely related recognized species.

Isolate	Closest species	% identity	Accession number of closest species
NP1	*B. subtilis*	98.76%	OP895017.1
NP2	*B. amyloliquefaciens*	99.65%	KF054918.1
NY1	*B. thuringiensis*	97.84%	KF779471.1
NY2	*B. pumilus*	98.27%	KT981882.1
NN1	*B. thuringiensis*	97.35%	KF779471.1
NN2	*B. pumilus*	96.94%	MW295357.1
NH1	*B. cereus*	97.41%	LK392517.1
NH2	*B. pumilus*	99.79%	EU231626.1
NC1	*Alcaligenes* sp.	80.14%	KP016627.1
NC2	*B. thuringiensis*	96.52%	KF779471.1

Comparative sequence analysis of the 16S rRNA gene indicated that the bacterial isolates were classified into two distinct genera. *Bacillus* spp. and *Alcaligenes* sp. NP1 is closely related to *B. subtilis* strain HAU427 (accession number OP895017.1), exhibiting 98.76% sequence homology. In contrast, NP2 is associated with *B. amyloliquefaciens* strain AR-NAW1-23 (accession number KF054918.1), demonstrating 99.65% sequence homology. The nearest strain for isolates NY1, NN1, and NC2 was identified as *B. thuringiensis* strain LDC507 (accession number KF779471.1). It exhibited 97.84%, 97.35%, and 96.52% identity with the three previously mentioned isolates, respectively. The 16S rRNA gene sequences for isolates NY2, NN2, and NH2 exhibited identities of 98.27%, 96.94%, and 99.79% with *B. pumilus* strain Suaeda B-006 (accession number KT981882.1), *B. pumilus* strain B2 (accession number MW295357.1), and *B. pumilus* strain TCCC111014 (accession number EU231626.1), respectively. The 16S rRNA of NH1 exhibited a similarity of 97.41% to *B. cereus* lSU-02 (accession number LK392517.1). NC1 exhibited an 80.14% relatedness to *Alcaligenes* sp. strain clone C1A04 (accession number KP016627.1) **(**Table 16).

Phylogenetic Analysis of Bacterial Isolates

A neighbor-joining phylogenetic tree constructed using the 16S rRNA gene sequences of closely related recognized species shows the taxonomic affiliation of our isolates. NP1, NP2, NY1, NY2, NN1, NN2, NH1, NH2, and NC2 were clustered with members of *Bacillus* spp. whereas NC1 formed a monophyletic subgroup with Alcaligenes sp. As shown in figure 9.

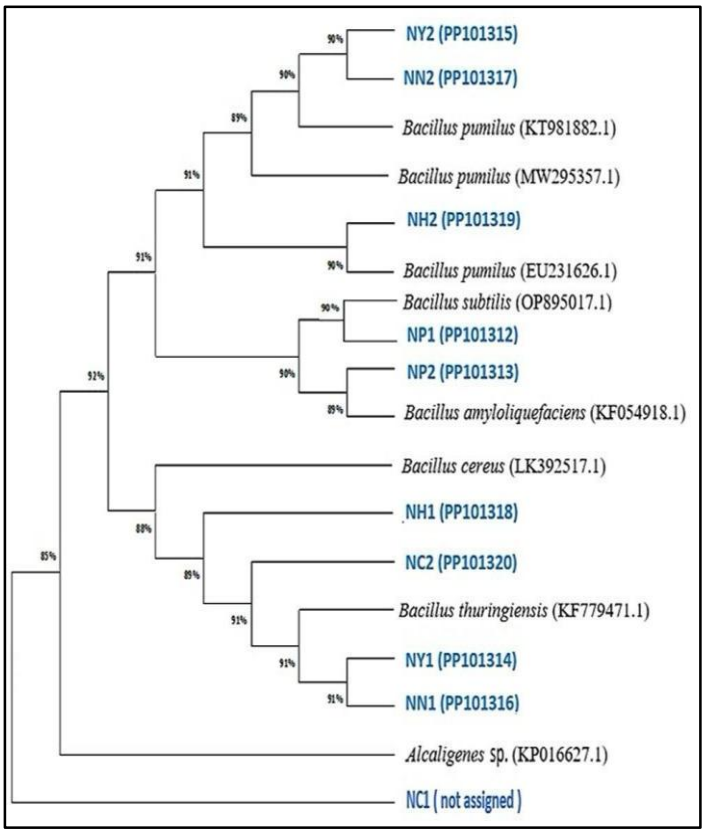


Figure 9 Phylogenetic tree by neighbor-joining method constructed of the ten isolates and other related isolates available in GenBank. A phylogenetic tree was constructed using MEGA7, version 11 software.

DISCUSSION

Global microorganism antibiotic resistance has arisen, exacerbated by the horizontal gene transfer of resistance genes between microbial species, which accelerates the spread of resistance mechanisms across diverse environments (Jeya et al., 2022).

More research on bacterial resistance and novel therapies are needed. In Al-Ahsa, Saudi Arabia, this study found antibiotic-producing bacteria, assessed their ability to inhibit pathogens and soil bacteria, and described them phenotypically and genotypically. From several soil samples, we isolated 14 bacteria. *S. enterica* were suppressed by NP1 and 2. NY1, NN1, NH1, and NC1 fight soil isolates. The ten isolates have distinct morphological and biological traits. The minimal inhibitory concentration (MIC) was determined using disc diffusion (0.75-12.5 µg/µl). Antibiotic-producing isolates developed secondary chemicals that inhibited dangerous bacteria. In a phylogenetic tree and comparative 16S rRNA gene sequence analysis, most isolates were *Bacillus* spp. Except NC1, which was *Alcaligenes* sp.

The collection of six soil samples from diverse environments including rhizosphere, water, non-agriculture region, poultry house, and dairy product farm in Al-Ahsa, Saudi Arabia provides an overview for exploring the diversity of antibiotic-producing bacteria, where each environment offers unique conditions that may influence the microbial population, composition, abundance, and activity **(Martiny *et al.*, 2006)**. For instance, the rhizosphere of *P. farcta* in Ayen Al-Harrah, a plant adapted to arid conditions, might harbor bacteria with unique stress-resistant mechanisms, potentially including novel antibiotic compounds to protect the plant from pathogens **(Valverde *et al.*, 2010)**. Similarly, Al-Asfar Lake and Al-Uqair represent aquatic environments, which are often rich in their soil microbial diversity but underexplored for antibiotic production **(Umeaku *et al.*, 2021).** In addition, the soil of circular road desert offers a baseline for understanding natural

soil bacterial communities and their antagonistic activities against each other without direct human influence **(Shinwari *et al.*, 2013)**. The poultry house soil and Nada dairy products farm soil may have been exposed to commercial antibiotics **(Herath *et al*., 2016; Burgos *et al*., 2005)**. However, these environments were chosen for the possibility of the discovery of new microbial species that may produce unique secondary metabolites that have antibiotic activity **(Church, 2022)**.

The isolation of bacteria from poultry house soil (2 isolates), Al-Asfar lake soil (3 isolates), Nada dairy products farm soil (4 isolates), the rhizosphere of *P. farcta* in Ayen Al-Harrah soil (2 isolates), circular road desert soil (2 isolates), and Al-Uqair beach soil (1 isolate) in Al-Ahsa, Saudi Arabia, may aid bioprospecting for novel antibiotics **(Geers *et al*., 2022)**.

Our primary screening of 14 isolates tested their pathogen and intergroup effectiveness. This stage identified isolates with antibiotic-producing morphologies and confirmed inhibitory zones around some colonies in the crowded plate technique **(Bhandare and Chavre, 2011)**. This method is used to examine antibiotic-producing microorganisms **(Bhandare and Chavre, 2011; Church, 2022)**. NP1 (*B. subtilis*) and NP2 (*B. amyloliquefaciens*) inhibited growth of *S. enterica*. This supports **Bala et al.**, **(2012)**, who reported that *Bacillus* sp. soil isolates suppress *Salmonella* sp**.** The global pathogen *S. enterica* can be inhibited by NP1 and NP2, suggesting they could be exploited to build novel multidrug-resistant drugs. Both NP1 and NP2 isolates inhibit *S. enterica* suggests developing drugs or chemicals that kill this pathogen **(Ebada and Ebrahim, 2020)**. Understanding these chemicals' processes may assist develop treatments with fewer adverse effects and microbiota disruption **(Kapoor *et al*., 2017)**. These isolates' environmental origins aid comprehension. Chicken farm antibiotic-producing isolates may have withstood intense microbial competition. **Church (2022)** suggested that antibiotics may boost secondary metabolite synthesis under competitive settings. Uninhibited pathogens like *E. Coli*, *S. aureus* and *C. albicans* by most isolates may demonstrate soil isolates' antibacterial specificity **(Ebada and Ebrahim, 2020)**. Inhibition assays on isolates from diverse Al-Ahsa settings showed exceptional competition and antibiotic synthesis **(Rana *et al*., 2019)**. NP2 and NY2 are inhibited by NY1, NN2 by NN1, NH2 by NH1, and NC2 by NC1, suggesting that these strains have antagonistic activities due to the production of secondary metabolites, especially antibiotics, which bacteria use to compete in their natural environments **(Rana *et al*., 2019).** Genetic diversity is shown by isolation inhibition patterns. Strains like NY1 and NH1 exhibited significant inhibitory effects, could indicate differences in their antibiotic-producing genes **(Wang *et al*., 2022)**. *B. thuringiensis* NY1 interacts uniquely with NP2 and NY2 isolates. These findings imply that NY1 may create compounds that inhibit NY2, a strain from the same habitat, and NP2, from a different site. As previously proven, *B. thuringiensis* may create strong chemicals that kill many bacteria **(Salazar-Marroquín *et al*., 2016)**. This inhibition may also suggest that NY1 kills bacteria in multiple ways **(Duportet *et al*., 2012)**. Understanding bacterial isolate interactions may reveal microbial community dynamics and lead to novel antibiotics **Mushtaq *et al*. (2023)**. The form, color, and borders of bacterial isolated colonies convey their identity and ecological adaptability. Many soil bacteria have circular colonies, indicating their ability to adapt to changing environmental circumstances necessary for survival in soil ecosystems **(Diabankana *et al*., 2022)**. Color changes in colonies may prevent UV and oxidative damage **(Reis-Mansur *et al*., 2019)**. Color differences may also indicate metabolic processes that create secondary metabolites like antibiotics **(van de Kerkhof *et al*., 2022**). The colonies' borders in NP1, NP2, and NN2 may show how these bacteria attach to surfaces, interact with other microorganisms, and respond to UV protection and nutrition intake **(Be'er et al., 2011)**. Disparities can disclose these bacteria's ecological roles and survival strategies in different environments. Gram staining provides the first information on bacterial cell wall composition, which is essential for drug and environmental interactions **(Mushtaq *et al*., 2023)**. Gram-positive bacteria dominated our study, with one Gram-negative. These data suggest Gram-positive bacteria predominating in soil types **(Mushtaq *et al*., 2023)**.

The API 20E test measures bacterial metabolism and enzymatic activity. It helps comprehend their functions in diverse situations **(Mohammad *et al*., 2020)**. The consumption of ONPG (ortho-nitrophenyl-β-galactoside) by isolates NY2, NN2, and NH2, unique urea hydrolysis activity in NN2, citrate (CIT) utilization in all isolates except NP1, NP2, and NC2, indole (IND) production by NC1, and gelatin hydrolysis by most isolates, except NY2 and NH2, may indicate metabolic pathway diversity due to enzymes. Different metabolic processes may produce bioactive molecules with antibacterial action. Recently, indole-containing compounds have been demonstrated to boost antibiotic efficacy due to their bacteria-fighting ability **(Ramkissoon *et al*., 2020)**. **Ramkissoon *et al*. (2020)** found three indole alkaloid compounds that inhibit Gram-positive bacteria like *S. aureus* and Gram-negative bacteria *S. enteritidis* from *P. aeruginosa*. *It has been reported that Enterococcus faecium* from chicken feces improves chicken immune response and alleviates *Salmonella* infections via possibly production of antibiotics **(Khalifa & Mohamed 2023)**. One of the most important antioxidant enzymes, catalase, converts H_2O_2 into oxygen and water to prevent toxicity **(Onajobi *et al*., 2020)**. Catalase is found in 90% of our isolates, suggesting an evolutionary tolerance to reactive oxygen species. This supports a recent investigation that found all *Bacillus* and *Alcaligenes* isolates catalase-positive **(Shan *et al*., 2024)**. Interestingly, catalase has indirect antibacterial activity, according to a comparative study of microbes on healthy skin and keloid disease-affected skin. A moderate increase in oxidative stress due to the absence of catalase in keloid skin bacteria increases their growth and worsens the disease. These bacteria increase keloid fibroblast development in patients. In contrast, healthy skin has catalase-positive bacteria such *B. subtilis*, avoid this sickness **(Shan *et al*., 2024)**. The absence of catalase activity in *B. subtilis* NP1 is noteworthy. NP1 may use alternate oxidative stress mechanisms because of this divergence **(Petruk *et al*., 2018)**. This supports **Petruk *et al*. (2018)**, where *B. subtilis* spores may help eukaryotic cells resist ROS-induced damage. Spore attachment to the infected cell induces NADPH oxidases, which increase ROS levels and nuclear transfer of Nrf-2, which binds to the receptors of genes that produce antioxidant enzymes like HO-1. Our isolates' morphological and biochemical variety may reveal soil bacterium diversity. Understanding the variety of metabolic pathways in these bacteria may lead to the discovery of new antibiotic secondary metabolites.

Extracting metabolites from bacterial isolates is essential for bioactive chemical discovery **(Pinu *et al*., 2017)**. Various solvents are used to solubilize different molecules in this stage **(Pinu *et al*., 2017)**. The quantity and diversity of extracted metabolites depend on solvent selection **(Pinu *et al*., 2017)**. Due to time constraints, we only used methanol in our study. Future research will compare the quantity and effect of alternative solvents in extracting antibacterial secondary metabolites. Methanol is used to extract microbial metabolites quickly and easily **(Pinu *et al*., 2017)**. It is also a good polar solvent for antibiotic extraction **(Pinu *et al*., 2017)**. Evaporating the sample at moderate temperatures removes it easily, making it suited for secondary metabolites that are sensitive to high temperatures **(Pinu *et al*., 2017)**.

Antimicrobial research relies on Minimum Inhibitory Concentration (MIC) to assess microbial extract potency against diverse bacteria **(Ramachandran *et al*., 2014)**. MIC values in crude extracts of isolates can vary from 0.78 to 12.5 µg/µl due to solvent type, concentration, and microorganism sensitivity (**Be'er et al., 2011; Carvalho and Van Der Sand., 2016)**. MIC values of NY1 (*B. thuringiensis*) crude extract was 6.15 µg/µl against NY2 (*B. pumilus*) and 0.78 µg/µl against NP2 (*B. amyloliquefaciens*), suggesting different antagonistic effects of crude extract components against these isolates **(Carvalho and Van Der Sand, 2016)**. Lower MIC values indicate that crude extract components affect NP2 more than NY2. The different sensitivity of NP2 and NY2 isolates to NY1 crude extract may explain this **(Carvalho and Van Der Sand, 2016)**. MIC values for crude extracts against *S. entrica* varied from 3.13 µg/µl in NP1 (*B. subtilis*) to 1.56 µg/µl in NP2 (*B. amyloliquefaciens*). Due to genetic diversity of these strains **(Ramachandran *et al*., 2014; Sitotaw et al. 2022)**, 16S rRNA gene sequencing may disclose the diversity or quantities of chemicals in bacterial crude extracts **Sitotaw et al. (2022)**. This pathogen's MIC values may represent its diversity of susceptibility to NP1 and NP2 extracts **(Ramachandran *et al*., 2014)**. **Ramachandran *et al*. (2014)** found a lower MIC of *B. subtilis* against several Gram-negative infections compared to this study. The MIC value of *B. amyloliquefaciens* crude extract was also found by **Lin *et al*. (2020)** against Gram-negative bacteria was lower than our results. Comparing these data may help explain how various bacterial strains express antimicrobial secondary metabolites depending on environmental circumstances and organism **(Sitotaw et al., 2022)**. Our investigation found a MIC value of 12.5 µg/µl for NH1 (*B. cereus*) crude extract against Gram-positive bacteria, compared to a recent study by **Amin *et al*. (2015)**. In the related study, it was 25 µg/µl **(Amin *et al*., 2015)**. The solvent, which was polar in our study and nonpolar in the related study, may have affected its ability to extract physiologically active molecules. Additionally, the studied organism's susceptibility to the extract varies.

Our investigation found that a single colony could not manufacture enough antibiotics to establish large inhibition zones, as revealed in primary screening. In contrast, more colonies could produce antibiotics. This supports a prior study that found a group of colonies can manufacture enough antibiotics to reach a bioactive concentration **(Subirats *et al*., 2023)**.

Gas Chromatography-Mass Spectrometry (GC-MS) is a powerful analytical method for identifying and quantifying compounds in microbial extracts, which often contain fatty acids, alcohols, esters, and more complex organic molecules **(Anantha *et al*., 2016)**. Our bacterial extracts from various environments in Al-Ahsa were analyzed using GC-MS, and the most prominent metabolites were Cyclohexasiloxane, dodecamethyl-, Cycloheptasiloxane, tetracadymethyl, phenol, and 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-. The ecological niches from which these bacteria originated may explain their variety. The bacterial isolate from Nada dairy products farm was exposed to nutrients and environmental stresses that characterize their habitats, which explains the presence of Cyclohexasiloxane, dodecamethyl, with 14.23% area and Cycloheptasiloxane, tetracadymethyl, with 10.27% area in NN1, which were higher than their areas in NC1. The metabolic characteristics of NN1 and NC1 from non-agricultural areas differ **(Church, 2022)**. **El-Fayoumy *et al*. (2023)** found significant amounts of two substances in *Nostoc linckia* crude extract, with 24.81% and 17%, respectively, and suspected antibacterial activity. Other chemicals in NC1 and NH1crude extracts include phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl- and 1,4-benzenedicarboxylic acid, and bis(2-ethylhexyl) ester. **Hidhayati *et al*. (2020)** found that *C. turgidus* crude extract had antibacterial properties may be caused by 1,2-benzenedicarboxylic acid, bis (2-)-Ethylhexyl ester, and phenolic substances.

A distinctive peak in the GC-MS spectra of docosanoic acid, ethyl ester in NY1 crude extract indicates that this chemical is an ester of lauric acid, which could be employed as an antibacterial and antioxidant **(Gurung *et al*., 2009)**. These bioactive chemicals may work synergistically in bacterial crude extracts to create powerful antibacterial activities **(Adeniyi *et al.*, 2016)**.

16S rRNA gene sequencing is a standard approach for bacterial identification and classification **(Janda and Abbott, 2007)**. Sequencing confirmed the taxonomic identities and phylogenetic relationships of nine isolates from soil samples in Al-Ahsa, Saudi Arabia, which were *Bacillus* spp. with one *Alcaligenes* sp. isolation. NP1 was *B. subtilis* with 98.76% sequence homology. NP2 as *B. amyloliquefaciens* with 99.65% sequence similarity. NY1, NN1, and NC2 were *B. thuringiensis* with 97.84%, 97.35%, and 96.52% sequence homology. The designation of NY2, NN2, and NH2 was *B. pumilus* and has 98.27%, 96.94%, and 99.79% sequence homology. NH1 had 97.41% homology to *B. cereus*. NC1 shared only 80.14% sequence homology with *Alcaligenes* sp. NC1 may represent a distinct species or subspecies in the Alcaligenes genus due to its decreased homology percentage, which could lead to new metabolic pathways and bioactive chemicals **(Zahir *et al*., 2013)**.

The predominance of *Bacillus* species in these samples may indicate their ability to adapt to diverse environmental conditions due to their broad physiological capabilities, such as secreting diverse groups of enzymes to exploit diverse carbon sources, which may lead to the production of several secondary metabolites with antimicrobial effects, and their ability to form spores, which allows them to survive in harsh environments. In comparison, *Alcaligenes* sp. is a widespread soil microorganism that uses many carbon sources for growth **(Ishaya *et al*., 2023)**. We only found one isolate of this genus in our investigation, possibly due to soil sample collection or incubation circumstances like temperature **(Ishaya *et al*., 2023)**. A neighboring phylogenetic tree employing 16S rRNA gene sequences clustered bacterial isolates with known taxa. Our taxonomic and phylogenetic results match our morphological and biochemical characterizations, verifying prior phenotypic characterization. *B. subtilis* (NP1) morphology, which are Gram-positive colonies, circular, creamy white, flat, with undulated borders, positive gelatin test, and negative indole test, comparable with **Yunus *et al*. (2016)**. According to **Soliman *et al*. (2022)** *B, amyloliquefaciens* (NP2) are Gram-positive, indole-negative, urease-negative, and gelatin-positive bacteria with round, creamy white colonies. *B. thuringiensis* (NY1), (NN1), and (NC2) had Gram-positive, circular, white, flat, and entire colony surfaces, identical to those previously described in **Bravo *et al*. (2007)**, as well as some biochemical test results with **Adwitiya *et al*. (2009)**, such as negative indole and urease results and positive citrate utilization results. *B. pumilus* (NY2), (NN2), and (NH2) were Gram-positive, round, and white colonies resembled **(Qureshi and Elhassan, 2016)**. **Menon *et al*. (2010)** noted their citrate- and sucrose-positiveness. *B. cereus* (NH1) was Gram-positive, circular, white, with entire margin colonies, and indole-negative, citrate-positive, and gelatin-positive, fermenting glucose, fructose, maltose, and sucrose, consistent with **(Hadi, 2019)**. Furthermore, biochemical and morphological traits of *Alcaligenes* sp. (NC1) were Gram-negative, circular, white, and convex colonies identical to those previously reported with the same species, including catalase production and urease absence (**Zahir *et al*., 2013)**.

Discovery of varied *Bacillus* species and maybe novel *Alcaligenes* isolates could lead to additional research into their ecological functions, metabolic capabilities, and antibiotic manufacturing applications.The findings underscore the ecological and industrial significance of soil-derived bacterial isolates from Al-Ahsa, Saudi Arabia, particularly their ability to produce bioactive compounds with antibacterial properties. These isolates, primarily from the *Bacillus* and *Alcaligenes* genera, demonstrate natural antagonism against pathogens like *Salmonella enterica*, highlighting the ecological role of soil microbiota in maintaining microbial balance and suppressing harmful microorganisms. Identified metabolites, such as cyclohexasiloxane and phenol derivatives, offer potential for antibiotic development, addressing the global threat of antimicrobial resistance. Industrially, these strains could be utilized in sustainable agriculture as biocontrol agents, in probiotic formulations for livestock to enhance immunity, or as cost-effective sources of novel antibiotics. While challenges such as high R&D costs, regulatory compliance, and scale-up processes exist, the benefits, including environmental sustainability, public health advancements, and economic gains, make these bacterial isolates valuable candidates for addressing pressing global issues like antibiotic resistance and agricultural sustainability.

CONCLUSION

This study tested fourteen Al-Ahsa, Saudi Arabia, bacterial strains for antibiotic synthesis. Six isolates showed antibacterial activity against *S. enterica* and each other. Bacterial isolates have varied phenotypes. Crude extracts showed antibacterial activity with MIC values from 0.78 to 12.5 µg/µl. Cyclohexasiloxane, dodecamethyl Cycloheptasiloxane, tetracadymethyl, and phenol derivatives, detected by GC-MS, may be bioactive compounds. Comparative 16S rRNA gene sequence analysis identified the isolates as *B. subtilis* NP1, *B. amyloliquefaciens* NP2, *B. thuringiensis* NY1, NN1, and NC2, *B. pumilus* NY2, NN2, and NH2, *B. cereus* NH1, and *Alcaligenes* sp.

Limitations and Future Directions:This study faced certain limitations, including the relatively small number of bacterial isolates tested and the need for further investigation into the mechanisms by which these bacteria produce antimicrobial compounds. Future research should focus on extracting secondary metabolites using a variety of polar and non-polar solvents to evaluate their effectiveness by comparing the diversity and quantities of metabolites in each crude extract, followed by testing these extracts against a range of pathogenic microbes. Additionally, the chemical structures of biologically active compounds produced by these strains should be identified using advanced techniques such as nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction. Further studies should also aim to determine the precise modes of action of these secondary metabolites, clarifying whether they exert inhibitory or lethal effects on microbial cells. In-depth characterization of antibiotic-producing bacterial strains, particularly NC1, which exhibited lower homology to *Alcaligenes* sp., using whole-genome sequencing and DNA-DNA hybridization, could help assign novel taxa to antibiotic-producing bacteria. Finally, identifying the secondary metabolic gene clusters responsible for antibiotic production will reveal the potential for the bioproduction of new biologically active compounds effective against pathogens.In vivo testing to evaluate their effectiveness in clinical settings.

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ABBREVAITION

API	Analytical Profile Index
ADH	Arginine dihydrolase
AMY	Amygdalin fermentation
ARA	Fermentation of arabinose
BLAST	Basic local alignment search tool
°C	Celsius
DNA	Deoxyribonucleic acid
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
IND	Indole
L	Liter
LDC	Lysine decarboxylation
MIC	Minimum inhibitory concentration
min	Minute
ml	Milliliter
NA	Nutrient Agar
NaCl	Sodium Chloride
NB	Nutrient broth
NCBI	National Centre for Biotechnology Information
ONPG	Ortho-nitrophenyl-β-galactoside
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ROS	reactive oxygen species
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S	Seconds
TDA	Tryptophan deaminase
VP	Voges-Proskauer
µg	Microgram
µg /µl	Microgram per microliter

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