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Original article

Production of antibacterial compounds using *Bacillus* spp. isolated from thermal springs in Saudi Arabia

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ABSTRACT

Seventeen water samples were collected from four different thermal springs in Saudi Arabia. Microbiological assays were used to assess the antibacterial activities of bacterial colonies against antibiotic-resistant and susceptible-bacterial strains, and 16S rRNA gene sequencing was used to identify the genus and species of these antibiotic-producing bacteria. Chromatography and spectroscopy were used to separate the active compounds and help figuring out what their structures were. Four compounds were isolated using bacteria: *N*-acetyltryptamine (**1**), isovaleric acid (**2**), ethyl-4-ethoxybenzoate (**3**) and phenylacetic acid (**4**). Compounds **1**, **2** and **4** were produced from *Bacillus pumilus* and **3** was from *Bacillus licheniformis* (AH-E1). The outcomes of the minimum inhibitory concentrations (MICs) showed that all pure compounds produced in this work had antibacterial activities against Gram-positive pathogens (between 128 mg/L and 512 mg/L compared to the control) and compound **2** had activity against *E. coli*.

1. Introduction

A considerable global health crisis that threatens communities has been attributed to the emergence of resistant bacterial infections (Khameneh et al., 2016). The dissemination of resistance within bacterial pathogens results in a gradual decline in the effectiveness of antibiotics over time (Rossolini et al., 2014). As a result, the search for novel antibiotics from different natural sources has increased (Alrumman et al., 2018). Some microorganisms are able to produce secondary metabolites for protection against competition, and bacteria are superlative at producing clinically valuable antibiotics.

One of the most critical and niche habitats for bacteria is geothermal springs, which have been used for spas as well as for treating dermatological infections (Hussein and Loni, 2011). In the Kingdom of Saudi Arabia, several thermal springs have been used for hundreds of years for both domestic and irrigation purposes. Examples include the Ghomygah and Al-Aridhah springs, and microorganisms that live in these habitats are either thermophilic or hyperthermophilic.

Because of their harsh environment, it is thought that thermophilic bacteria are a biodiverse reservoir to produce bioactive secondary metabolites (Alrumman et al., 2018). Thermophiles also produce numerous valuable products such as antibiotics, thermostable enzymes, and anticancer compounds, making this source of considerable global interest (Salem et al., 2016; Shakhatreh et al., 2017).

Thermophilic *Bacillus* have the ability to produce a large number of extra cellular thermostable enzymes that have considerable industrial importance including lipases, amylases, cellulases and proteases (Lele and Deshmukh, 2016). Furthermore, several *Bacillus* species are able to create various secondary metabolites which are diverse in function and structure (Athukorala et al., 2009). For example, peptide antibiotics such as gramicidins, bacitracins, and tyrocidines are produced by members of *Bacillus* (Esikova et al., 2002).

B. paralicheniformis is a facultative anaerobic, motile Gram-positive bacterium, which is closely related to *B. sonorensis* and *B. licheniformis* based on phylogenetic analysis (Du et al., 2019). Fengycin is a peptide antibiotic drug that has been discovered from *B.*

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paralicheniformis (Du et al., 2019) and the lantibiotic formicin is also a peptide produced by *B. paralicheniformis* (Collins et al., 2016).

Both *B. licheniformis* and *B. paralicheniformis* have been utilized for years in the biotechnology sector in order to synthesize antibiotics, enzymes as well as consumer and biochemical products (Du et al., 2019). Moreover, they both widely used commercially for plant protection, as live organisms in feed applications and in aquaculture (Agersø et al., 2019).

Four hot springs in Saudi Arabia, some of which had never been studied before, were selected in this work to isolate bacterial strains capable of producing antibacterial metabolites and evaluate their biological activity as antimicrobial agents.

2. Materials and methods

2.1. Sample collection and bacterial isolation

Water samples were collected in June 2016 from four different hot springs in Saudi Arabia. Each sample was collected in a 20 mL sterile tube. Thirteen samples were collected from the Aseer region, KSA, and four from the Jazan region, KSA. All were transported to the School of Pharmacy at UCL for further work and stored at 4 °C until analysis.

2.1.1. Growth and isolation of bacteria

1 mL of each water sample was filtered using a 0.22 μm syringe filter (microfiltration) into a new reaction tube and then 150 μL of each was transferred onto a blood agar plate (Oxoid, UK) and spread using a disposable cotton swab. Subsequently, plates were incubated at 37 °C for 72 h (Genlab). After incubation, each colony with a different color or morphology was collected with a disposable sterile loop and streaked onto a new blood agar plate, and stored at 4 °C until analysis.

2.1.2. Bacterial culture

Each isolated bacterium was transferred onto a blood agar plate and streaked in different directions in order to ensure its purity and to make a bacterial culture, which was used as a stock culture.

2.2. Bacterial identification

2.2.1. Extraction of genomic DNA from isolated bacteria

Four bacterial strains were cultured on blood agar (each strain on one plate) and incubated at 37 °C for 18 h preparation for DNA extraction. The DNeasy® UltraClean® Microbial Kit (Qiagen, Germany) was used for DNA extraction according to the manufacturer's protocol. The isolated DNA was analyzed by gel electrophoresis and nanodrop (Thermo) to ensure of purity and measure its concentration, before being stored at 4 °C for further analysis.

2.2.2. Biochemical tests and molecular identification

Isolates were identified at the genus level using motility test, Gram stain, endospore test, catalase test, oxidase test, thermotolerance test and anerobic growth test (Holt et al., 1994). The 16S rRNA sequence was used for identification at the species level. Four isolated DNA samples from different bacterial strains were subjected to PCR to amplify them for 16S rRNA gene sequencing and the primers are detailed in Table 1. PCR amplification was performed according to the conditions recommended by the manufacturer for the Phusion High-Fidelity DNA Polymerase (New England Biolabs – Massachusetts, USA). Each bacterial colony was suspended in 1 mL of distilled water prior to analysis. The reaction mixture for each sample (50 μL) contained: 1 μL of the isolated DNA, 10 μL of a 5X Phusion buffer, 1 μL of dNTPs (10 mM), 0.5 μL of each primer (FD1 + RP1) (10 μM), 0.5 μL of Phusion polymerase and 36.5 μL of microbiological H_2O. Reaction conditions for amplification were 94 °C for 5 min, 35 cycles [94 °C for 30 secs, 62 °C for 30 secs, 72 °C for 30 secs], and 72 °C for 5 min (Bio Rad). The PCR products (a little bit above 1517 bp) were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. All tubes were then analyzed by gel electrophoresis and nanodrop and stored at 4 °C for further analysis. Phylogenetic analysis was performed using MEGA X software, a multiple alignment of 12 partial sequence of 16S rRNA closely matches isolates were retrieved from GenBank and were aligned using Clustal W software. *E. coli* was used as outgroup.

Table 1
Primers used in this work.

Primer	Sequence	Primer site	Reference
Forward PCR Primer (FD1)	5′-AGAGTTTGATCCTGGCTCAG-3′ (Epicenter Technologies – Wisconsin, USA)	8–27	Devereux and Willis, 1995
Reversed PCR Primer (RP1)	5′-ACGGTTACCTTGTTACGACTT-3′ (Epicenter Technologies – Wisconsin, USA)	1512–1492	

2.2.3. 16S rRNA gene sequencing

30 ng/μL from each pure PCR product was prepared in EB solution for sequencing. All samples were sent to the UCL Sequencing Facility for 16S rRNA gene sequencing using the following primers: FD1 and RP1. The chromatograms of obtained nucleotide sequences of PCR products were manually assessed for quality using SnapGene Viewer 3.0.1. (GSL Biotech LLC, USA). Quality was assessed using Bioedit 7.2.5. (Ibis Biosciences, USA). The sequence data was saved as a FASTA file and then converted to a translated sequence and compared for homology using BLASTx (https://blast.ncbi.nlm.nih.gov, National Center for Biotechnology Information, USA).

2.3. Extraction and isolation of active compounds

2.3.1. General experimental procedures

IR spectra were measured on a Perkin Elmer 100 FT-IR spectrometer. 1D ^{1}H, ^{13}C and DEPT-135, and 2D HMQC, HMBC, COSY and NOESY NMR spectroscopic data were acquired on a Bruker Avance 500 MHz NMR spectrometer. Deuterated solvents and NMR tubes were purchased from Cambridge Isotope Laboratories and Sigma-Aldrich, respectively. Bruker Topspin, version 3.2 software was used to process NMR data. Low-resolution mass spectra were acquired on a LCQ Duo Ion-Trap mass spectrometer (Thermo Fisher Scientific). Column chromatography was performed on silica gel 60 (0.04–0.063 mm; Merck) and TLC on Silica gel 60 F254 (Merck) plates. Vanillin-sulfuric acid reagent was used to visualize the TLC plates. All chemicals used were HPLC grade. Chemicals were supplied by either Sigma-Aldrich or Fisher Scientific.

2.3.2. Solid-state fermentation

One single colony was inoculated onto one blood agar plate in order to be cultured. The plate was then incubated at 37 °C for 24 h. After incubation, colonies were transferred into a reagent bottle containing 30 mL of sterile PBS as a stock solution. Then, each 1 mL was transferred into a vial containing 20 mL of PBS. 1 mL was then inoculated onto one blood agar plate and incubated for one week at 37 °C. 600 plates were used as a large-scale

fermentation for each bacterium and 20 blood plates were used as control.

2.3.3. Extraction and isolation of bioactive compounds

After seven days of incubation, the agar was cut into small cubes (approximately 1 cm in length of each side) and scraped away and transferred into a conical flask (500 mL). (3 × 300 mL) of chloroform was first utilized to kill bacteria as well as to extract compounds from the agar. Next, (3 × 300 mL) of methanol and (3 × 300 mL) of water were used to extract the active compounds, respectively. All extractions were performed by agitation on a rotatory shaker (IKA) at 160 rpm/min, at 37 °C for 24 h**.** Subsequently, all extracts were sonicated (Grant) at 40 °C for 30 min and filtered into a round-bottom flask. Chloroform and methanol extracts were evaporated using a rotatory evaporator (Buchi). However, the water extract was evaporated via freeze-drying (Savant). All extracts were weighed and kept at 4 °C until analysis.

2.3.4. Separation of active compounds by TLC and bioautography

Active fractions from extracts were applied on a normal or reverse phase TLC plate (5 × 10 cm) in order to separate the active compound. Three solvent systems were used for this method: the first (*n*-hexane: ethyl acetate: methanol) (80:20:1) was used for the fractions of AH-E1. For the fractions of TH-C4, TB-A3 and KH-A1, (chloroform: methanol) (90:10) was used. 50 mg from each fraction was dissolved in 1 mL of a suitable solvent (methanol or chloroform) and then 5 drops from each were spotted onto the TLC plate at least 1 cm from its bottom edge. Next, the TLC plate was developed in a twin trough glass chamber saturated with the mobile phase solvents for 30 min up to a distance of 80 mm. After solvent development, the plate was visualized under UV TLC visualizer at UV254 nm and UV366 nm to ensure that the separation was of sufficient quality. The TLC plate was then directly placed into a sterile cabinet for drying for at least one hour, and then transferred into a sterile petri dish. A bacterial inoculum of *S. aureus* with a density of 5×10^5 CFU/mL was prepared and added to 20 mL of molten nutrient agar. After gentle shaking (to avoid air bubbles), the molten agar was poured over the TLC plate and incubated for 18 h at 37 °C. After incubation, the plate was treated with a 1 mg/mL solution of MTT in methanol and incubated for 30 min at 37 °C to reveal zones of inhibition. Living bacteria were seen as a purple color, whereas a yellow zone indicated an active compound able to inhibit the bacterial growth.

2.4. Antibacterial assay

2.4.1. Test organisms

Five Gram-positive and three Gram-negative bacteria were used in this project as test organisms: *Staphylococcus aureus* (ATCC-25923), SA 1199B, eMRSA-15, XU212, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

2.4.2. Broth dilution assay

A bacterial inoculum of each test organism with a density of 5×10^5 CFU/mL was prepared. 51.2 mg from each extract was dissolved in 1 mL of DMSO and then 10 μL of this solution was dissolved in 500 μL of broth for the MIC assay. Pure DMSO was used as the control. A 96-well plate (Thermo Scientific) was used and 200 μL of each sample was transferred to the first column of this plate. 100 μL of MH broth was pipetted into the wells from column 2 to column 9 in order to be used for serial dilution. 100 μL from each well in the first column was then transferred to the second column and mixed well. This step was repeated until column 9. After that, the excess amount was transferred to column 12 as a sterile control for each sample. 100 μL of a test organism was added to column 11 to be used as a growth control. Norfloxacin (Fluka BioChemika) was used as a positive control in row 8 with all tested strains except for tetracycline, which was used with SA 1199B strain. 2% of DMSO was added to column 10 to ensure it had no positive effect on inhibition of bacterial growth. MHB was added to row 7 as a blank. 100 μL of the tested strain was added to each well and then the plate was incubated at 37 °C for 18 h. After the incubation, 20 μL of a 1 mg/mL solution of MTT in methanol was added to each well and incubated for 30 min at 37 °C. A blue color indicated growth and a yellow color indicated a lack of growth. MIC values were recorded as the lowest concentration at which no color change was observed. The MIC experiment was done in duplicate per plate and repeated in at least three independent experiments.

3. Results

3.1. Isolation and identification of bacterial strains

In this study, strains **KH-A1**, **TB-A3**, **AH-E1** and **TH-C4** (Table 2) were isolated from four different springs in Saudi Arabia. The strains were named according to the spring source that they were isolated from. For example, the first two letters are from the spring's name, the third letter from the sample's name and the numbers indicate the colony number from each sample. Bacterial identification showed that all isolates were classified as closely of *Bacillus* genus (Table S7). The findings of the 16S rRNA gene sequencing illustrated those strains **KH-A1** (97.79%), **TB-A3** (97.09%) and **TH-C4** (97.48%) had the highest homology with *Bacillus pumilus*. Strain **AH-E1** had the highest homology with *Bacillus licheniformis* (97.02%) (Table S6). According to phylogenetic analysis of 16S rRNA gene (Fig. 1), it was possible to identify each strain at the species level.

3.2. Isolation and identification of active compounds

Four known compounds (**1**–**4**) (Fig. 2) were isolated from the different bacterial strains. **1** was isolated from the methanol extract of **KH-A1** and identified as *N*-acetyltryptamine based on its NMR, MS and IR spectral data (Table S1), which correlated well with the published ^{13}C data of Zhang et al., (2013). The molecular formula of **1** was determined as $C_{12}H_{14}N_2O$ and the molecular weight of the compound was calculated as 202, which corresponded to a pseudo molecular ion peak at *m*/*z* 225.13 $[M+Na]^+$ in its ESI-MS.

Natural product **2** was isolated from the methanol extract of strain **TB-A3** and its molecular formula was determined as $C_5H_{10}O_2$ on the basis of a pseudo molecular ion at at *m*/*z* 101.0 $[M-H]^-$ in its ESI-MS. It was readily identified as isovaleric acid by comparison of its NMR spectral data (Table S2) with that published (Bal et al., 2008).

Compound **3** was isolated from the methanol extract of the **AH-E1** strain and identified as ethyl-4-ethoxybenzoate based on the findings of its NMR (Siddique, 2019), MS and IR spectral data (Table S3). The molecular formula of **3** was determined as $C_{11}H_{14}O_3$ and the molecular weight of the compound was calculated as 194, which was supported by an ion in the ESI-MS at *m*/*z* 195.10 $[M+H]^+$.

Secondary metabolite **4** was isolated from a methanol extract of strain **TH-C4** and identified as phenylacetic acid based on its NMR (Holmes and Lightner, 1995), MS and IR spectral data (Table S4). Its molecular formula was confirmed as $C_8H_8O_2$ with a molecular weight of 136, which corresponded to an ion in the ESI-MS at *m*/*z* 135.04, attrributable to $[M-H]^-$.

Table 2
Bacterial isolates from each spring.

Spring	Location	Antibiotic-Producing Bacteria
Tharban spring (TH)	183 Km northwest of Abha city, (41°67**E**-19°03**N**)	TH-C4
Al-Khobh spring (KH)	81 Km southeast of Jazan city, (43°07**E**- 16°45**N**)	KH-A1
Al-Haridhah spring (AH)	132 Km west of Abha city, (42°05**E**- 18°01**N**)	AH-E1
Thebah spring (TB)	158 Km southeast of Abha city, (43°15**E**- 17°62**N**)	TB-A3

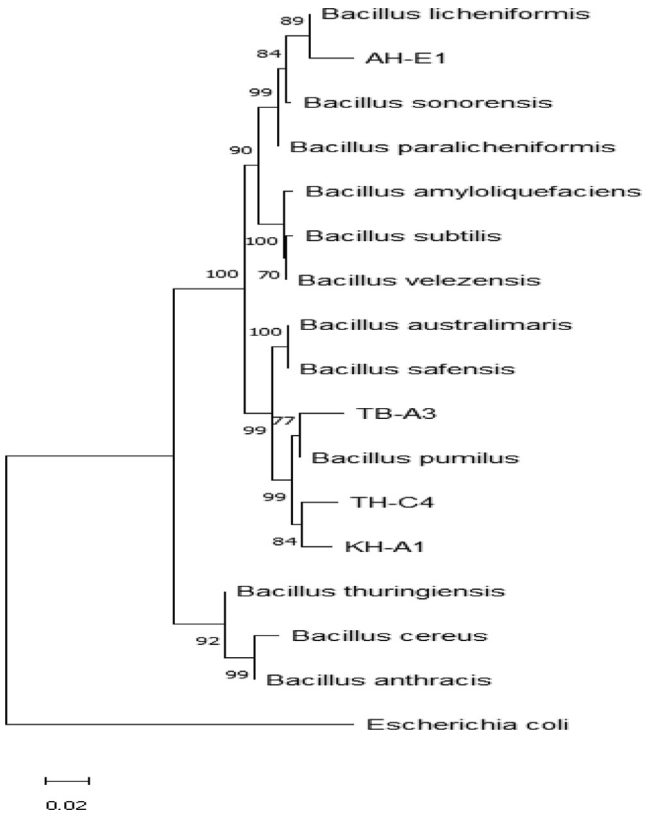

Fig. 1. Comparative analysis of partial 16S rRNA gene sequences of the isolates obtained from thermal springs in Saudi Arabia. The phylogenetic tree was inferred using the neighbor-joining method with a bootstrap analysis of 1000 repetitions.

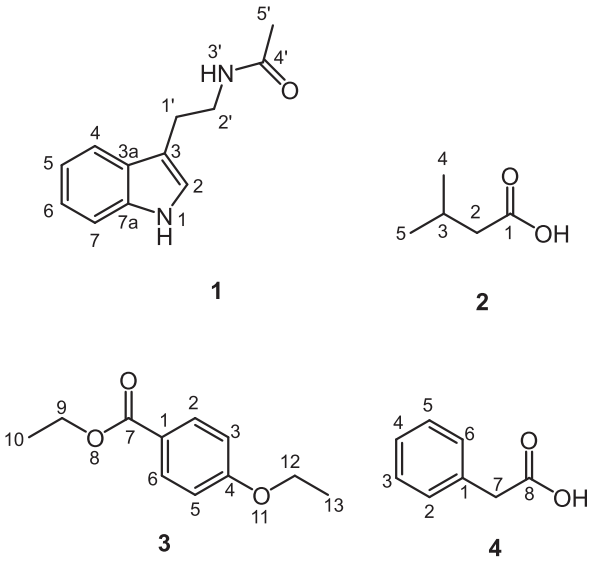

Fig. 2. Structures of **1**–**4**.

3.3. Antibacterial activity of isolated compounds

As illustrated in Table 3, compounds **1**, **3** and **4** had antibacterial activity (narrow-spectrum activity) against only the Gram-positive bacteria. In contrast, compound **2** illustrated moderate broad-spectrum activity toward both Gram-positive and Gram-negative strains.

4. Discussion

Bacillus spp. are one of the most prominent genera of thermophilic bacteria that have been studied (Lele and Deshmukh, 2016). This genus currently comprises 377 species and can be found in aquatic environments, soil, food as well as in the gut microbiota of mammals and arthropods (Caulier et al., 2019). *Bacillus* spp. are known as rod-shaped, Gram-positive bacteria (Sumi et al., 2015). Additionally, *Bacillus* can be found singly or in pairs, and in chains or as long filaments (Logan and Vos, 2015) and the thermophilic bacilli may survive better at temperatures between 45 and 70 °C (Adiguzel et al., 2009).

B. licheniformis has been discovered from Tharban (Alrumman et al., 2018) and Al-Khobh springs (Yasir et al., 2019), but this is the first time it was isolated from the Al-Haridhah spring. Furthermore, *B. pumilus* has been previously found in the Al-Khobh spring (Yasir et al., 2019). However, its discovery from the Tharban and Thebah springs is reported here for the first time.

Several studies have recorded the isolation of *N*-acetyltryptamine from various microbes, for example, from different bacteria such as *Corallococcus corraloides* (Böhlendorf et al., 1996), *Intrasporangium* strain N8 (Okudoh and Wallis, 2012), *Streptomyces* spp. (AC-2) (Lin et al., 2008), *Streptomyces* spp. strain TN58 (Mehdi et al., 2009), *Streptomyces djakartensis* (Zhang et al., 2013) and *Streptomyces* spp. MBT76 (Wu et al., 2016). Furthermore, it has also been found in some fungi, including *Penicillium vitale* (Vinokurova et al., 2000), *Penicillium solitum* (Antipova et al., 2018) and *Fusarium poae* (Nagia et al., 2012). It has also been isolated from a *Micromonospora* spp. strain associated with the marine sponge *Tethya aurantium* (Tuan et al., 2017) as well as from *Microbispora aerata* from Antarctica (Bratchkova and Ivanova, 2011). In *Bacillus* spp., *B. cereus* readily produces *N*-acetyltryptamine as a metabolite of tryptamine (Hutzinger, 1969), but it is reported here for the first time from *B. pumilus*.

Numerous studies have described the isolation of isovaleric acid from microbes. For example, it has been isolated from *Peptostreptococcus anaerobius* (Guerrant et al., 1982), *Pseudoalteromonas haloplanktis* strain (Hayashida-Soiza et al., 2008) and *Megasphaera hexanoica* spp. *nov* (Jeon et al., 2017). Furthermore, it has also been isolated from *Bacillus* spp. including *B. amyloliquefaciens* (Chen et al., 2017), *B. subtilis* (Hong et al., 2017; Mumtaz et al., 2019), *B. velezensis* (Calvo et al., 2020), *B. cereus* and *Paenibacillus polymyxa* (Mumtaz et al., 2019).

In this study, isovaleric acid was isolated from *B. pumilus* from a Saudi spring, which supports a study stating that *B. pumilus* was able to produce isovaleric acid (de la Cochetière-Collinet and Larsson, 1984). In general, isovaleric acid and other simple acids such as isocaproic and 2-methylbutyric acids are thought to be

Table 3
MIC values of (**1**–**4**) and standard antibiotics in μg/mL.

	S. aureus	SA-1199B	eMRSA-15	XU212	*B. subtilis*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
1	128	256	256	512	>512	>512	>512	>512
2	256	256	512	512	512	512	>512	>512
3	256	512	512	>512	>512	>512	>512	>512
4	128	256	256	512	512	>512	>512	>512
Nor[a]	1	–	1	16	1	1	1	1
Tet[b]	–	16	–	–	–	–	–	–

[a] Norfloxacin.
[b] Tetracycline.

induced by L-leucine catabolism in anaerobic bacteria (Díaz-Pérez et al., 2016).

Ethyl-4-ethoxybenzoate has been isolated from both plants and microbes. From microbes, it has been isolated from the hexane extract of *B. licheniformis* (Sharma et al., 2010) as well as from *Paenibacillus* spp. (Tahir et al., 2019).

Ethyl-4-ethoxy benzoate was isolated from *B. licheniformis* from the Al-Haridhah spring and this is consistent with the study of Sharma et al., (2010), who isolated the same compound from this species.

There are numerous studies on the isolation of phenylacetic acid from microbes. For instance, it has been isolated from *Streptomyces zhaozhouensis* (Lacret et al., 2015), *Streptomyces humidus* (Hwang et al., 2001), *Proteus mirabilis* (Erdmann, 1987), *Azospirillum brasilense*, *Enterobacter cloacae*, *Thauera aromatica* and the nitrogen-fixing bacteria *Frankia* (Cook, 2019), *Bacteroides ruminicola, Bacteroides melaninogenicus* subsp. *macacae*, *Clostridium botulinum* type G and in other species of *Clostridium* (Mayrand and Bourgeau, 1982). Moreover, phenylacetic acid has been produced by the fungi such as *Rhizoctonia solani*, *Sporobolomyces roseus* and *Schizophyllum commune* (Cook, 2019). It has also been found in the brown alga, *Undaria pinnatifida* (Cook, 2019). Furthermore, it has been reported that phenylacetic acid was found in some *Bacillus* species. For example, *B. fortis* (Akram et al., 2016), *B. megaterium*, *B. cereus* strain HY-3 and *B. subtilis* strain HY-16 (Hwang et al., 2001) as well as *B. licheniformis* (Kim et al., 2004).

This is the first report of phenylacetic acid being produced by *B. pumilus*. However, it has been isolated from some *Bacillus* spp. such as *B. licheniformis* (Kim et al., 2004) and *B. cereus* (Hwang et al., 2001). Moreover, it was previously identified in the same spring (Tharban), but from a different organism, *Streptomyces* spp. (Al-Dhabi et al., 2019). Phenylacetic acid (also known as benzene acetic acid or α-toluic acid) has drawn the attention of the pharmaceutical industry due to its various biological properties such as antioxidant, anti-inflammatory and antifungal properties as well as its ability to inhibit quorum sensing in *P. aeruginosa* (Musthafa et al., 2012). It has a prevalent distribution in nature, and formed metabolically from L-phenylalanine (Burkhead et al., 1998).

In comparison with the literature, *N*-acetyltryptamine has been reported to have antibacterial activity against *B. subtilis*, *B. cereus*, *S. aureus*, *E. coli,* MRSA and *Pseudomonas syringae pv. actinidiae* (Zhang et al., 2013). Furthermore, it has been published as an antifungal and antibacterial agent with activity against *Fusarium* spp., *Verticillium dahlia* and the Gram-positive bacterium *Micrococcus luteus* (Mehdi et al., 2009).

Moreover, it has been reported that isovaleric acid has antibacterial properties (Hayashida-Soiza et al., 2008; Huang et al., 2011). Ethyl-4-ethoxybenzoate has been described as an anti-biofilm agent (Campbell et al., 2019). In addition, phenylacetic acid has been shown to demonstrate antibacterial and antifungal activities against *E. coli*, MRSA, *A. fumigatus* and *C. albicans*, (Lacret et al., 2015). Moreover, it demonstrated antimicrobial activity against *E. coli* and *Ralstonia solanacearum* (Zhu et al., 2011), *S. aureus, E. coli*, *C. albicans*, *Pseudomonas aeruginosa* and *B. subtilis* (Kim et al., 2004). It has also been found as an antibacterial agent (Erdmann, 1987) and antifungal compound (Hwang et al., 2001).

5. Conclusion

In conclusion, seventeen water samples were collected from four different hot springs in Saudi Arabia. Several biological and microbiological assays were used to assess the antibacterial activity of samples against resistant- and susceptible-bacterial strains, and to identify the genus of the bacteria. Four antibacterial-producing bacterial strains were isolated from these springs and identified as *Bacillus licheniformis* (one strain) and *Bacillus pumilus* (three strains). Additionally, four compounds were produced using these bacteria: *N*-acetyltryptamine, isovaleric acid, ethyl-4-ethoxybenzoate and phenylacetic acid. These compounds have been isolated from these springs for the first time except phenylacetic acid. The findings of this study revealed that *Bacillus* spp., isolated from hot springs in Saudi Arabia, could be used to produce a variety of antibacterial compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2023.05.015.

References

Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M., Sahin, F., 2009. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. J. Microbiol. Methods 79 (3), 321–328.

Agersø, Y., Bjerre, K., Brockmann, E., Johansen, E., Nielsen, B., Siezen, R., Stuer-Lauridsen, B., Wels, M., Zeidan, A.A., Chang, Y.-F., 2019. Putative antibiotic resistance genes present in extant *Bacillus licheniformis* and *Bacillus paralicheniformis* strains are probably intrinsic and part of the ancient resistome. PLoS One 14 (1), e0210363.

Akram, W., Anjum, T., Ali, B., 2016. Phenylacetic acid is ISR determinant produced by *Bacillus fortis* IAGS162, which involves extensive re-modulation in metabolomics of tomato to protect against *Fusarium wilt*. Front. Plant Sci. 7, 1–12.

Al-Dhabi, N.A., Esmail, G.A., Duraipandiyan, V., Arasu, M.V., 2019. Chemical profiling of *Streptomyces* spp. Al-Dhabi-2 recovered from an extreme environment in Saudi Arabia as a novel drug source for medical and industrial applications. Saudi J. Biol. Sci. 26. 4, 758–766.

Alrumman, S., Mostafa, Y.S.M., Al-Qahtani, S., Taha, T.H.T., 2018. Hydrolytic enzyme production by thermophilic bacteria isolated from Saudi hot springs. Open Life Sci. 13 (1), 470–480.

Antipova, T.V., Zhelifonova, V.P., Baskunov, B.P., Kochkina, G.A., Ozerskaya, S.M., Kozlovskii, A.G., 2018. Exometabolites the *Penicillium* fungi isolated from various highlatitude ecosystems. Microbiol. 87 (5), 642–651.

Athukorala, S.N., Fernando, W.D., Rashid, K.Y., 2009. Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Canadian J. Microbiol. 55 (9), 1021–1032.

Bal, D., Kraska-Dziadecka, A., Gradowska, W., Gryff-Keller, A., 2008. Investigation of a wide spectrum of inherited metabolic disorders by 13C NMR spectroscopy. Acta Biochim. Pol. 55 (1), 107–118.

Böhlendorf, B., Bedorf, N., Jansen, R., Trowitzsch-Kienast, W., Höfle, G., Forche, E., Gerth, K., Irschik, H., Kunze, B., Reichenbach, H., 1996. Antibiotics from gliding bacteria, LXXIII indole and quinoline derivatives as metabolites of tryptophan in myxobacteria. Liebigs Ann. 1996 (1), 49–53.

Bratchkova, A., Ivanova, V., 2011. Bioactive metabolites produced by microorganisms collected in Antarctica and the Arctic. Biotech. Biotech. Equipment 25 (sup1), 1–7.

Burkhead, K.D., Slininger, P.J., Schisler, D.A., 1998. Biological control bacterium *Enterobacter cloacae* S11: T: 07 (NRRL B-21050) produces the antifungal compound phenylacetic acid in Sabouraud maltose broth culture. Soil Biol. Bioch. 30 (5), 665–667.

Calvo, H., Mendiara, I., Arias, E., Gracia, A.P., Blanco, D., Venturini, M.E., 2020. Antifungal activity of the volatile organic compounds produced by *Bacillus velezensis* strains against postharvest fungal pathogens. Postharvest Biol. Tech. 166, 1–10.

Campbell, M., Zhao, W., Fathi, R., Mihreteab, M., Gilbert, E.S., 2019. *Rhamnus prinoides* (gesho): A source of diverse anti-biofilm activity. J. Ethnopharm. 241, 1–9.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front. Microbiol. 10 (302), 1–19.

Chen, Y., Liu, M., Chen, S., Wei, X., 2017. Decreased formation of branched-chain short fatty acids in *Bacillus amyloliquefaciens* by metabolic engineering. Biotechnol. L. 39 (4), 529–533.

Collins, F.W., O'Connor, P.M., O'Sullivan, O., Rea, M.C., Hill, C., Ross, R.P., 2016. Formicin–a novel broad-spectrum two-component lantibiotic produced by *Bacillus paralicheniformis* APC 1576. Microbiol. 162 (9), 1662–1671.

Cook, S.D., 2019. An historical review of phenylacetic acid. Plant Cell Physiol. 60 (2), 243–254.

De la Cochetière-Collinet, M.F., Larsson, L., 1984. Studies on volatile metabolites of some potentially pathogenic *Bacillus* species, using automated head-space gas chromatography. J. Chromat. B: Biomed Sci. Applic. 305, 178–182.

Devereux, R., Willis, S.G., 1995. Amplification of ribosomal RNA sequences. Molec. Microb. Ecol. Man., 277–287

Díaz-Pérez, A.L., Díaz-Pérez, C., Campos-García, J., 2016. Bacterial l-leucine catabolism as a source of secondary metabolites. Rev. Environ. Sci. Bio/Technol. 15 (1), 1–29.

Du, Y., Ma, J., Yin, Z., Liu, K., Yao, G., Xu, W., Fan, L., Du, B., Ding, Y., Wang, C., 2019. Comparative genomic analysis of *Bacillus paralicheniformis* MDJK30 with its closely related species reveals an evolutionary relationship between *B. paralicheniformis* and *B. licheniformis*. BMC Genomics 20 (1).

Erdmann, G.R., 1987. Antibacterial action of myiasis-causing flies. Parasitol. Today 3 (7), 214–216.

Esikova, T.Z., Temirov, Y.V., Sokolov, S.L., Alakhov, Y.B., 2002. Secondary antimicrobial metabolites produced by thermophilic *Bacillus* spp. strains VK2 and VK21. Applied Bioch. Microbiol. 38. 3, 226–231.

Guerrant, G.O., Lambert, M.A., Moss, C.W., 1982. Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. J. Clin. Microbiol. 16 (2), 355–360.

Hayashida-Soiza, G., Uchida, A., Mori, N., Kuwahara, Y., Ishida, Y., 2008. Purification and characterization of antibacterial substances produced by a marine bacterium *Pseudoalteromonas haloplanktis* strain. J. Appl. Microbiol. 105 (5), 1672–1677.

Holmes, D.L., Lightner, D.A., 1995. Synthesis and acidity constants of 13CO2H labelled mono and dipyrrole carboxylic acids. pKa from 13C-NMR. Tetrahedron 51 (6), 1607–1622.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of Determinate Bacteriology. Williams and Wilkins.

Hong, C., Chen, Y., Li, L., Chen, S., Wei, X., 2017. Identification of a key gene involved in branched-chain short fatty acids formation in natto by transcriptional analysis and enzymatic characterization in *Bacillus subtilis*. J. Agricul. Food Chem. 65 (8), 1592–1597.

Huang, C.B., Alimova, Y., Myers, T.M., Ebersole, J.L., 2011. Short-and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. Archi. Oral Biol. 56 (7), 650–654.

Hussein, M.T., Loni, O.A., 2011. Major ionic composition of Jizan thermal springs, Saudi Arabia. J. Emerg. Trends Engin. Appl. Sci. 2. 1, 190–196.

Hutzinger, O., 1969. *N*-acetyltryptamine: An artifact formed during the extraction of tryptamine. Anal. Biochem. 30 (1), 153–156.

Hwang, B.K., Lim, S.W., Kim, B.S., Lee, J.Y., Moon, S.S., 2001. Isolation and in vivo and in vitro antifungal activity of phenylacetic acid and sodium phenylacetate from *Streptomyces humidus*. Appl. Enviro. Microbiol. 67 (8), 3739–3745.

Jeon, B.S., Kim, S., Sang, B.I., 2017. *Megasphaera hexanoica sp. nov*., a mediumchain carboxylic acid-producing bacterium isolated from a cow rumen. Intern. J. Systematic and Evolut. Microbiol. 67. 7, 2114–2120.

Khameneh, B., Diab, R., Ghazvini, K., Bazzaz, B.S.F., 2016. Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. Microb. Pathog. 95, 32–42.

Kim, Y., Cho, J.Y., Kuk, J.H., Moon, J.H., Cho, J.I., Kim, Y.C., Park, K.H., 2004. Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus licheniformis* isolated from fermented soybean, Chungkook-Jang. Curr. Microbiol. 48 (4), 312–317.

Lacret, R., Oves-Costales, D., Gómez, C., Díaz, C., de la Cruz, M., Pérez-Victoria, I., Vicente, F., Genilloud, O., Reyes, F., 2015. New ikarugamycin derivatives with antifungal and antibacterial properties from *Streptomyces zhaozhouensis*. Mar. Drugs 13 (1), 128–140.

Lele, O.H., Deshmukh, P.V., 2016. Isolation and characterization of thermophilic *Bacillus* sp. with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India. Intern. J. Appl. Res. 2. 5, 427–430.

Lin, Z.J., Lu, X.M., Zhu, T.J., Fang, Y.C., Gu, Q.Q., Zhu, W., 2008. GPR12 Selections of the metabolites from an endophytic *Streptomyces* spp. associated with *Cistanche deserticola*. Archi. Pharm. Res. 31. 9, 1108–1114.

Logan, N.A., Vos, P.D., 2015. *Bacillus.* Bergey's Manual of Systematics of Arch. and Bac., 1–163.

Mayrand, D., Bourgeau, G., 1982. Production of phenylacetic acid by anaerobes. J. Clin. Microbiol. 16 (4), 747–750.

Mehdi, R.B.A., Shaaban, K.A., Rebai, I.K., Smaoui, S., Bejar, S., Mellouli, L., 2009. Five naturally bioactive molecules including two rhamnopyranoside derivatives isolated from the *Streptomyces* sp. strain TN58. Nat. Prod. Res. 23 (12), 1095–1107.

Mumtaz, M.Z., Barry, K.M., Baker, A.L., Nichols, D.S., Ahmad, M., Zahir, Z.A., Britz, M. L., 2019. Production of lactic and acetic acids by *Bacillus* sp. ZM20 and *Bacillus cereus* following exposure to zinc oxide: A possible mechanism for Zn solubilization. Rhizosphere 12, 1–10.

Musthafa, K.S., Sivamaruthi, B.S., Pandian, S.K., Ravi, A.V., 2012. Quorum sensing inhibition in *Pseudomonas aeruginosa* PAO1 by antagonistic compound phenylacetic acid. Current Microbiol. 65 (5), 475–480.

Nagia, M.M., Shaaban, M., Abdel-Aziz, M.S., El-Zalabani, S.M., Hanna, A.G., 2012. Secondary metabolites and bioactivity of two fungal strains. Egyptian Pharmaceut. J. 11 (1), 16–21.

Okudoh, V.I., Wallis, F.M., 2012. Enhanced recovery and identification of a tryptaminerelated antibiotic produced by *Intrasporangium* N8 from KwaZulu-Natal, South Africa. Tropic. J. Pharmaceut. Res. 11 (5), 729–737.

Rossolini, G.M., Arena, F., Pecile, P., Pollini, S., 2014. Update on the antibiotic resistance crisis. Curr. Opin. Pharmacol. 18, 56–60.

Salem, M.M.E., Ayesh, A.M., Gomaa, M.N.E.D., Abouwarda, A.M., 2016. Isolation, and identification of thermophilic bacteria from Al-Laith hot springs and detection of their ability to produce protease enzyme. Am.-Eurasian J. Agricu. Enviro. Sci. 16 (7), 1227–1236.

Shakhatreh, M.A.K., Jacob, J.H., Hussein, E.I., Masadeh, M.M., Obeidat, S.M., Abdulsalam, F.J., Abd Al-razaq, M.A., 2017. Microbiological analysis, antimicrobial activity, and heavy-metals content of Jordanian Ma'in hot-springs water. J. Infect. Public Health 10 (6), 789–793.

Sharma, P.K., Goel, M., Dureja, P., Uniyal, P.L., 2010. Isolation and identification of secondary metabolites from hexane extract of culture filtrate of *Bacillus licheniformis* MTCC 7445. Archi. Phytopath. Plant Protect. 43 (16), 1636–1642.

Siddique, H., 2019. Bangladeshi Medicinal Plants: Ethnopharmacology, Phytochemistry and Anti-Staphylococcal Activity. University of East London. Doctoral dissertation.

Sumi, C.D., Yang, B.W., Yeo, I.C., Hahm, Y.T., 2015. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Canadian J. Microbiol. 61 (2), 93–103.

Tahir, A.A., Mohd Barnoh, N.F., Yusof, N., Mohd Said, N.N., Utsumi, M., Yen, A.M., Hashim, H., Mohd Noor, M.J.M., Akhir, F.N.MD., Mohamad, S.E., Sugiura, N., Othman, N., Zakaria, Z., Hara, H., 2019. Microbial diversity in decaying oil palm empty fruit bunches (OPEFB) and isolation of lignin-degrading bacteria from a tropical environment. Microbes Enviro. 34 (2), 161–168.

Tuan, C.D., Huong, D.T.M., Ngan, T.B., Quyen, V.T., Minh, L.T.H., Brian, M., Minh, C.V., Cuong, P.V., 2017. Secondary metabolites from *Micromonospora* sp. (G044). Vietnam J. Sci. Techno. 55 (3), 251.

Vinokurova, N.G., Ozerskaya, S.M., Zhelifonova, V.P., Adanin, V.M., 2000. Taxonomic position and nitrogen-containing secondary metabolites of the fungus *Penicillium vitale* pidoplichko et bilai apud bilai. Microbiol. 69 (3), 343–346.

Wu, C., Zhu, H., Van Wezel, G.P., Choi, Y.H., 2016. Metabolomics-guided analysis of isocoumarin production by *Streptomyces species* MBT76 and biotransformation of flavonoids and phenylpropanoids. Metabol. 12 (5), 1–11.

Yasir, M., Qureshi, A.K., Khan, I., Bibi, F., Rehan, M., Khan, S.B., Azhar, E.I., 2019. Culturomics-based taxonomic diversity of bacterial communities in the hot springs of Saudi Arabia. OMICS: J. Integ. Biol. 23 (1), 17–27.

Zhang, W., Wei, S., Zhang, J., Wu, W., 2013. Antibacterial activity composition of the fermentation broth of *Streptomyces djakartensis* NW35. Molecules 18 (3), 2763–2768.

Zhu, Y.-J., Zhou, H.-T., Hu, Y.-H., Tang, J.-Y., Su, M.-X., Guo, Y.-J., Chen, Q.-X., Liu, B.o., 2011. Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2- phenylacetic acid. Food Chem. 124 (1), 298–302.