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Antibacterial Activity and Characterization of Bacteria Isolated from Diverse Types of Greek Honey against Nosocomial and Foodborne Pathogens

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Abstract: It has been suggested that microorganisms present in honey are a potential source of antimicrobial compounds. This study aimed to isolate and characterize bacteria from 46 Greek honey samples of diverse botanical and geographical origin and to determine whether these bacteria demonstrate antibacterial activity against five important nosocomial and foodborne pathogens. In total, 2014 bacterial isolates were obtained and screened for antibacterial activity. Overall, 16% of the isolates inhibited the growth of *Staphylococcus aureus*, 11.2% inhibited the growth of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, 10.2% inhibited the growth of *Salmonella* Typhimurium and 12.4% of the isolates affected the growth of *Citrobacter freundii*. In total, 316 isolates that inhibited the growth of more than two of the tested pathogens were grouped by restriction fragment length polymorphisms (RFLP) analysis of the 16S rRNA gene amplicon. Fifty of them were identified by 16S rRNA gene sequencing. The majority, 62% of the isolates, belonged to the genus *Bacillus*. Only 10% of the isolates were identified as Gram-negative bacteria. Furthermore, in several bacterial isolates, genes encoding polyketide synthases and nonribosomal peptide synthetases that catalyze the biosynthesis of secondary metabolites which might contribute to the exerted antimicrobial activity, were detected. This study demonstrates that honey microbiota exerts antimicrobial activity and is a putative source of secondary metabolites against important nosocomial and food pathogens that warrants further investigation.

Keywords: honey; microbiota; antimicrobial activity; 16S-rRNA gene; nonribosomal peptide synthetase; polyketide synthase; secondary metabolites

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1. Introduction

Honey is a natural substance produced by honeybees (*Apis mellifera* L.). Honeybees collect nectar (floral honey) or secretions of living parts of plants (honeydew honey) [1–3] and transform them by adding secretions from their own salivary glands [4]. It is a complex aqueous mixture, consisting of more than 200 compounds, mainly carbohydrates. Moreover, it contains small amounts of proteins, aminoacids, phytochemicals, and plant- and bee-derived enzymes such as glucose oxidase and catalase [5,6]. It is characterized by low water activity (aw = 0.50–0.60), low humidity (<18%), and high acidity (pH 3.4–6.1) [7,8]. The physicochemical and biological properties of honey depend on its botanical and geographical origin [9–11].

For centuries honey has been utilized not only as a food, but also as a medicine and food preservative [12,13]. Recent studies confirmed its beneficial bioactivity on human

health [11,14,15]. Antibacterial activity is the most investigated biological property of honey [16,17] and it is attributed to different factors [2]. Honey is a hostile environment for the survival and growth of different microorganisms, due to its physicochemical properties [8,12,18]. Hydrogen peroxide (H_2O_2) is one of the major antimicrobial factors in honey. It is generated by the bee-derived enzyme glucose oxidase (GOX) or via an alternative non-enzymatic pathway comprising polyphenolic compounds [6,17,19]. The presence of various phytochemical compounds is also important for the direct antimicrobial potential of honey [20]. Other components responsible for the antibacterial activity exerted by honey are the peptide bee defensin-1 [11,16], methylglyoxal (MGO), which is the main antimicrobial substance of manuka honey [14,21], and other unknown components [22].

Despite the various antibacterial factors [17], several studies have reported the isolation of microorganisms from honeys produced in diverse geographical locations [4,7,12,18]. Microorganisms in honey might be derived from primary and secondary sources. Primary sources include pollen, flowers, nectar, honeydew secretions [5,22,23], the environment (dust, air, soil) [7], as well as the honeybees (gut microbiota) or other insects [24–26]. Furthermore, microorganisms can be inoculated to honey during its extraction and post-harvest processing [7,23]. They are often related to hygiene and storage conditions [3,27]. Usually, they are yeasts, molds, and bacterial species (especially spore-forming) [18,24,28].

Honey microbiome has been suggested as a potential source of antimicrobial compounds [19,28]. Honey bacterial isolates produce antimicrobial compounds in vitro, but there is no direct evidence of such compounds present in honey [29]. Many secondary metabolites of high pharmaceutical and biotechnological interest are synthesized by non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) [30,31]. These enzymes are large multifunctional complexes organized as modules and are implicated in the biosynthesis of nonribosomal peptides (NRPs) and polyketides (PKs) respectively. These two large classes of natural products exert various biological properties, such as antimicrobial, anticancer, and immunosuppressive activity [32,33].

The aim of this study was to isolate and characterize bacteria from honeys of diverse botanical origin and locations across Greece, exhibiting antibacterial activity against five important nosocomial and foodborne pathogens. Furthermore, the presence of genes encoding PKSs and NRPSs, that might be implicated in the synthesis of antimicrobial compounds, has been investigated.

2. Materials and Methods

2.1. Honey Samples

A total of forty-six Greek honey samples from various geographical and botanical origins were provided by individual beekeepers and beekeeping associations. Each sample was assigned to a unique reference number. Details regarding botanical source, geographical location, and harvest date were recorded (Table S1). The identification of the botanical source of honeys was based on the flora availability during the harvest season, the location of the apiary and, in several cases, palynological analysis. Honey samples were stored at room temperature in dark conditions.

2.2. Bacterial Isolation

Each honey sample (500 µL) was diluted into 1.5 mL sterile Mueller Hinton (MH) Broth (25%, *v/v*) (NEOGEN, Heywood, UK) and mixed well.

Aerobic mesophilic bacteria: 100 µL of each suspension were plated in triplicate onto standard plate count agar (PCA) (Condalab, Madrid, Spain) [7,8] or Luria Bertani (LB) agar (Lab M, Heywood, UK) [12] and then incubated aerobically at 30 °C for 5 days. After incubation, in order to obtain pure bacterial cultures, single colonies were transferred onto new PCA Petri dishes using the successive subculturing method. Pure cultures were stored as glycerol stocks (MH broth +20% glycerol) in 96-well plates at −80 °C, until further analysis.

Aerobic spore-forming bacteria: each diluted sample was subjected to heat activation at 80 °C for 10 min and cooled at room temperature for another 15 min [8,18]. Then, 100 µL of each sample were spread in triplicate onto the selective *Bacillus cereus* medium (BCM) (Lab M, Heywood, UK) and incubated under aerobic conditions at 30 °C for 48 h. Pure cultures, obtained as described above, were stored as glycerol stocks (MH broth +20% glycerol) in 96-well plates at −80 °C, until further analysis.

Lactic acid bacteria (LAB): A 100 µL aliquot of each diluted sample was spread in triplicate onto the selected media, Man Rogosa and Sharpe (MRS) agar (NEOGEN, Heywood, UK) and incubated anaerobically in an AnaeroJar AG25 with the AnaeroGen Atmosphere Generation system (Oxoid, Basingstoke, UK) at 30 °C for approximately 4 days. Resazurin sodium salt (Alfa Aesar, Thermo FisherGmbH, Kandel, Germany) (30 mg/lt) was used as an indicator for anaerobic conditions (turns from blue to pink). Each colony was subcultured anaerobically onto MRS agar supplemented with 1% calcium carbonate ($CaCO_3$) (Scharlau, Barcelona, Spain) [32]. A clear zone around a colony indicates hydrolysis of $CaCO_3$ due to organic acid production [34]. Colonies that exhibited a clear zone were picked, cultured onto eppendorfs filled to the top with Thioglycolate Broth (Scharlau, Barcelona, Spain) +20% glycerol and stored at −80 °C, until further analysis.

2.3. Pathogenic Bacterial Strains

The antimicrobial activity of the bacterial isolates from different honeys was tested against methicillin-resistant *Staphylococcus aureus* (*S. aureus*) strain 1552, carbapenem-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) strain 1773, *Acinetobacter baumannii* (*A. baumannii*), *Citrobacter freundii* (*C. freundii*), and *Salmonella* Typhimurium (*S.* Typhimurium) strains. All strains were isolated from clinical samples, identified, and characterized by standard laboratory methods at Attikon University General Hospital [13,35]. All bacteria were routinely grown in MH Broth (NEOGEN, Heywood, UK) or MH agar (NEOGEN, Heywood, UK) at 37 °C.

2.4. Dual Culture Overlay Assay

The antibacterial activity was tested by dual culture overlay assay as previously described [28,36] with few modifications. A replicate of each 96-well plate was transferred onto PCA square Petri dishes (120 × 120 mm, Aptaca Spa, Canelli, Italy) by using a microplate replicator (Boekel Scientific, Pennsylvania, PA, USA) and incubated at 30 °C for 24 h. Then, each pathogen (approximately 9×10^4 cfu/mL) was mixed into soft Nutrient Agar (Biolab, Budapest, Hungary) containing 0.75% (*w/v*) agar, keeping it liquid at 42 °C. The mixture of soft agar and bacterial cells was poured as a thin overlayer on top of the plates with the overnight-cultivated replica of the bacterial isolates. The plates were incubated at 37 °C for 24 h and inhibition zones were observed. Each bacterial isolate that exhibited a clear inhibition zone was tested again, in triplicate, in order to confirm the antibacterial activity.

2.5. Grouping and Identification of Bacteria Exerting Antibacterial Activity

From the glycerol stock each isolate was plated on PCA agar and incubated aerobically at 30 °C for 24 h. A single colony was obtained and cultured in MH broth aerobically at 30 °C, overnight. Pure broth cultures were used for genomic DNA extraction with the ExtractMe Genomic DNA Kit (Blirt, Gdańsk, Poland). Universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) [37] and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [38] (Eurofins Genomics, Germany) were used to amplify the 16S rRNA gene by PCR. The reaction mixture contained: 1 U FastGene Taq DNA Polymerase (NIPPON Genetics, Tokyo, Japan), 1× PCR buffer A, 25 pmol of each primer, 1 mM dNTPs, 3 µL DNA template, and deionized sterile water to a final volume of 50 µL. The thermal cycler Primus 25 (PEQLAB Biotechnologie, Erlangen, Germany) was used in the following PCR conditions: initialization at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s,

annealing at 50 °C for 30 s, and elongation at 72 °C for 2 min. A final elongation step at 72 °C for 2 min was added.

Restriction fragment length polymorphisms analysis (RFLP) of 16S rRNA amplicons was conducted as a simple prescreening for dereplication and grouping of the bacterial isolates. Two µL of each PCR product (approximately 1400 bp), were incubated at 37 °C for 1 h, with 10 U of *RsaI* and *HinfI* endonucleases and 10 × buffer K (Bioron, Römerberg, Germany) [5,18,25]. The analysis was performed by electrophoresis in a 3% agarose gel (Invitrogen, Paisley, UK) by standard electrophoresis. Following RFLP analysis, bacterial isolates from each group were phenotypically characterized by Gram staining [39].

Amplicons of representative isolates of each group demonstrating antibacterial activity were purified using the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and then directly sequenced via Sanger dideoxy termination method by Macrogen Europe (Amsterdam, The Netherlands). Chromas version 2.6.6 software (Technelysium Pty Ltd., South Brisbane, Australia, www.technelysium.com.au) (accessed on 21 June 2021) was used to check the quality of the obtained sequencing results. Sequences were assembled into a single sequence via MEGA X version 10.1.6 software [40] and Gene Runner version 6.5 software (www.generunner.net accessed on 21 June 2021) and subjected to a BlastN (Megablast) (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 21 June 2021, BLAST, NIH) search in the 16S rRNA Database-GENEBANK to identify sequences with the highest similarity [3,5,18]. Obtained sequences were deposited to GenBank under the following accession numbers: MW700012–MW700061.

2.6. NRPS and PKS Gene Screening

Identified bacterial isolates were further screened for the presence of biosynthetic gene clusters of nonribosomal peptide synthetases (NRPS) and type-I polyketide synthases (PKS-I). The oligonucleotide primer set NRPS-1F (5′-GCSTACSYSATSTACACSTCSGG-3′) and NRPS-1R (5′-SASGTCVCCSGTSCGGTAS-3′) (Eurofins Genomics, Ebersberg, Germany) was used to amplify partially the adenylation (A) domain (approximately 750–800 bp) and the primer set PKS-IF (5′-GTGCCGGTNCCRTGNGYYTC-3′) and PKS-IR (5′-CGATGGAYCCNCARCARYG-3′) (Eurofins Genomics, Ebersberg, Germany) for the amplification of partially KS domain (approximately 600–750 bp) [32]. PCR reactions were carried out in 50 µL final volume, using Primus 25 thermal cycler (PEQLAB Biotechnologie, Erlangen, Germany). The reaction mixture for NRPS A domain contained 1.5 U FastGene Taq DNA Polymerase (NIPPON Genetics, Tokyo, Japan), 1× PCR buffer B, 25 pmol of each primer, 1 mM dNTPs and 5 µL DNA template. The amplification conditions were the following: initialization at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 2 min and elongation at 72 °C for 4 min with a final elongation step at 72 °C for 1 min. For the KS domain the reaction mixture contained 1 U Taq DNA Polymerase (Invitrogene, UK), 1× PCR buffer, 25 pmol of each primer, 1 mM dNTPs, 1.5 mM $MgCl_2$, and 5 µL DNA template. The initialization at 94 °C for 3 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and elongation at 72 °C for 30 s. The final elongation step was performed at 72 °C for 5 min. Sequencing was performed via Sanger dideoxy termination method by CeMIA SA (Larissa, Greece). Sequencing data were subjected to a BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi, BLAST, NIH) (accessed on 21 June 2021) search and to BLASTX (E value: 1×10^{-5}) in order to identify the closest known homologues and determine the level of homology of the amino acid sequence.

3. Results

3.1. Isolation of Bacteria from Honey

In total, 2014 bacterial isolates were obtained from 46 Greek honey samples of various geographical and botanical origin, utilizing four different culture media (PCA, LB agar, BCM and MRS agar) (Table S1). Overall, 695 bacterial isolates were obtained on PCA, 851 on LB agar, 370 on BCM, and finally 98 isolates were obtained by using MRS agar. Bacterial

isolates were obtained from all honey samples no matter the botanical source, geographical origin, and the date of harvest. The only exception was honey sample 42, which was tested only for aerobic spore-forming bacteria on BCM.

3.2. Antimicrobial Activity of Bacterial Isolates

All bacterial isolates (2014 in total) were screened by dual overlay assay, in order to determine whether they inhibited the growth of five important human and foodborne pathogens: *S. aureus*, *P. aeruginosa*, *S.* Typhimurium, *A. baumannii*, and *C. freundii* (Table 1). A clear inhibition zone (>5 mm) against a given tested pathogen was considered as positive antibacterial activity. Overall, 323 (16%) isolates were found to inhibit the growth of *S. aureus*, 225 (11.2%) isolates inhibited the growth of *P. aeruginosa*, 206 (10.2%) isolates inhibited the growth of *S.* Typhimurium, while 225 (11.2%) isolates and 249 (12.4%) isolates affected the growth of *A. baumannii*, and *C. freundii*, respectively. Collectively, 296 (14.7%) isolates inhibited the growth of at least one of the tested pathogens, 167 (8.3%) isolates inhibited the growth of at least two of the five tested pathogens and 111 (5.5%) honey bacterial isolates affected the growth of at least three of the five tested pathogens. Forty (1.99%) isolates exerted antibacterial activity against at least four out of five tested pathogens, whereas 21 isolates (1.04%) inhibited the growth of all five pathogens. These results demonstrated that 31.5% (635/2014) of the obtained bacterial isolates exerted antibacterial activity against at least one pathogen.

3.3. Grouping and Identification of Bacterial Isolates Exerting Antibacterial Activity

The bacterial isolates (316) that exerted antibacterial activity against at least two of the tested pathogens were selected for further analysis. The 16S rRNA gene of all these isolates was amplified by PCR and the amplicons were subjected to RFLP analysis. Overall, the 316 bacterial isolates were distributed in 19 groups (Table S2). Representative isolates from each group were further subjected to Gram staining. In total, 50 bacterial isolates were identified by 16S rRNA gene sequencing.

Sequencing of the 16S rRNA gene revealed that 90% of the 50 bacterial isolates were classified as Gram-positive bacteria whereas only 10% as Gram-negative. Thirty-one out of 50 strains (62%) belonging to the genus *Bacillus* were classified into eight species (Table 2). *B. safensis* was the most frequently identified species (7 out of 31 isolates, 22.6%) followed by *B. pumilus* (5/31, 16.1%), *B. subtilis* (4/31, 12.9%), and *B. cereus* (2/31, 6.5%). Four isolates were identified as *B. licheniformis*, *Priestia megaterium* (former *B. megaterium*), *B. paramycoides*, and *B. vallismortis* respectively. Classification at species level within the *Bacillus* group was not possible for 9 isolates due to the high similarity of 16S rRNA gene sequence to more than one species. Other isolates were identified as *Lysinibacillus fusiformis*, *Microbacterium imperiale*, *Micrococcus yunnanensis*, *Paenibacillus* sp., *Paenibacillus profundus*, *Terribacillus saccharophilus*, and *Terribacillus* sp. (2% for each phylotype). Seven isolates were identified as *Staphylococcus* spp. (14%). Specifically, five isolates were identified as *S. arlettae*, *S. epidermidis*, *S. hominis*, *S. pasteuri*, and *S. warneri* respectively, and two isolates were identified as *S. cohnii*. Regarding the Gram-negative isolates, four (8%) were identified as *Pseudomonas* sp., *P. coleopterorum*, *P. fulva*, and *P. stutzeri* and one as *Acinetobacter lwoffii* (Table 2).

Table 1. Growth inhibition of selected human and foodborne pathogens exerted by bacterial isolates from various honeys.

Sample No	*S. aureus* [1]	*P. aeruginosa* [1]	*S.* Typhimurium [1]	*A. baumannii* [1]	*C. freundii* [1]	1/5 [2]	2/5 [2]	3/5 [2]	4/5 [2]	5/5 [2]	Total	%
1	25	23	26	6	25	23	17	12	3	-	55/127	43.3
2	8	7	5	7	8	11	6	4	-	-	21/77	27.3
3	17	9	7	10	12	19	10	4	1	-	34/118	28.8
4	33	19	11	23	22	26	14	14	3	-	57/112	50.9
5	18	8	9	9	12	15	10	4	1	1	31/90	34.4
6	17	8	3	4	5	12	8	3	-	-	23/70	32.9
7	15	15	8	11	18	24	12	5	1	-	42/117	35.9
8	5	5	7	8	7	7	9	1	1	-	18/62	29.0
9	8	3	1	4	4	12	4	-	-	-	16/81	19.8
10	13	6	11	5	5	17	6	2	-	1	26/83	31.3
11	8	5	4	10	6	9	6	1	1	1	18/56	32.1
12	7	4	4	3	6	6	2	2	2	-	12/41	29.3
13	18	7	9	11	14	22	5	6	1	1	35/112	31.3
14	35	15	19	25	27	51	22	6	2	-	81/190	42.6
15	4	3	5	5	4	-	-	2	-	3	5/19	26.3
16	3	2	3	2	2	-	2	1	-	1	4/30	13.3
17	1	1	1	2	-	-	1	1	-	-	2/27	7.4
18	6	7	7	8	6	3	1	2	2	3	11/38	28.9
19	1	1	-	1	-	-	-	1	-	-	1/7	14.3
20	2	3	2	3	1	-	-	1	2	-	3/20	15.0
21	3	7	8	9	4	-	6	5	1	-	12/38	31.6
22	2	2	2	2	1	-	1	1	1	-	3/24	12.5
23	3	3	3	2	4	1	1	4	-	-	6/9	66.7
24	3	3	3	1	3	4	1	1	1	-	7/36	19.4
25	-	-	-	-	-	-	-	-	-	-	0/22	0,0
26	11	5	-	2	2	9	4	1	-	-	14/40	35.0
27	2	2	1	1	2	5	-	1	-	-	6/43	14.0
28	-	1	-	-	-	1	-	-	-	-	1/28	3.6
29	17	17	15	22	13	2	4	7	7	5	25/57	43.9
30	5	2	1	1	2	2	3	1	-	-	6/42	14.3
31	-	-	-	1	1	-	1	-	-	-	1/29	3.4
32	1	2	3	5	3	2	-	1	1	1	5/34	14.7
33	2	4	1	5	3	2	2	3	-	-	7/37	18.9
34	1	1	1	1	1	-	-	-	-	1	1/1	100
35	3	5	4	3	4	4	1	-	2	1	8/10	80.0
36	2	4	3	2	3	1	-	3	1	-	5/17	29.4
37	-	-	1	-	1	-	1	-	-	-	1/1	100
38	1	1	1	1	-	-	-	-	1	-	1/2	50.0
39	-	1	-	-	-	1	-	-	-	-	1/4	25.0
40	1	-	-	-	-	1	-	-	-	-	1/2	50.0
41	10	10	8	6	9	1	5	5	3	1	15/17	88.2
42	-	-	-	-	-	-	-	-	-	-	0/0	-
43	1	-	-	2	-	3	-	-	-	-	3/27	11.1
44	-	-	-	-	-	-	-	-	-	-	0/1	0.0
45	11	4	9	2	9	-	2	6	2	1	11/14	78.6
46	-	-	-	-	-	-	-	-	-	-	0/2	0.0
Total	323	225	206	225	249	296	167	111	40	21	635/2014	
			Total %			14.7	8.3	5.5	1.99	1.04		31.5

[1] Columns demonstrate the number of bacterial isolates per honey sample that exerted activity against the corresponding pathogens. [2] Total number of bacterial isolates that inhibited the growth of 1–5 pathogens.

Table 2. Molecular identification of bacterial isolates and molecular screening for the presence of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes.

Group	Strain	bp	% Identity	Antibacterial Activity [1]	Bacteria	Accession Number	NRPS	PKS
A	CTA1	1381	100	1,2,3,4,5	*B. pumilus*	MW700012	+ *	-
	CTA2	1395	100	1,2,3,4,5	*B.pumilus*	MW700013	+	-
	CTA15	1376	100	2,3,5	*B.pumilus*	MW700019	+	-
	CTA16	1389	99.6	2,3,5	*B.vallismortis*	MW700020	+	+ *
	CTA31	1347	100	1,2,5	*Bacillus* sp. (*B. aerius*/*B. altitudinis*/ *B. aerophilus*/*B. stratosphericus*)	MW700025	+	-
	CTA57	1385	100	2,3,4	*B.safensis*	MW700028	+	+
	CTA146	1419	100	4,5	*B.safensis*	MW700038	-	+
	CTA163	1311	99.9	3,5	*B.licheniformis*	MW700039	+ *	-
	CTB7	1400	100	1,2,3,4,5	*B.safensis*	MW700041	-	+
	CTB16	1403	100	1,2,3,4,5	*B.safensis*	MW700043	+	-
	CTB21	1300	100	1,2,3,4	*B.pumilus*	MW700044	+	-
	CTB26	1340	99.9	1,2,4,5	*B.pumilus*	MW700045	-	-
	CTB31	1094	99.9	1,3,4,5	*Bacillus* sp. (*B. amyloliquefaciens*/*B. velezensis*)	MW700046	+	+ *
	CTB43	1403	100	2,3,4	*B.safensis*	MW700049	+	+
	CTB89	1132	100	1,5	*B.safensis*	MW700053	+	+
	CTB120	1369	100	3,4	*B.safensis*	MW700057	+	-
B	CTA5	1396	99.9	1,2,3,5	*B.subtilis*	MW700014	+	+ *
	CTA9	1384	100	2,3,4,5	*B.subtilis*	MW700016	+	+ *
	CTA10	1373	100	2,3,4,5	*B.subtilis*	MW700017	+ *	+ *
	CTA14	681	99.6	2,3,5	*Bacillus* sp. (*B. haynesii*/*B. piscis*/ *B. paralicheniformis*/*B. licheniformis*)	MW700018	+ *	-
	CTA20	1410	100	1,3,5	*B. subtilis*	MW700021	+ *	+ *
	CTA109	1414	99.9	2,5	*Bacillus* sp. (*B. halotolerans*/*B. mojavensis*)	MW700035	-	+ *
	CTB11	1281	99.6	1,2,3,4,5	*Bacillus* sp. (*B. subtilis subsp. Inaquosorum*/ *B. nakamurai*/*B. mojavensis*/ *B. halotolerans*)	MW700042	-	+ *

Table 2. *Cont.*

Group	Strain	bp	% Identity	Antibacterial Activity [1]	Bacteria	Accession Number	NRPS	PKS
C	CTA7	1401	99.9	1,3,4,5	*P. megaterium* (*B. megaterium*)	MW700015	+ *	-
	CTA28	1401	100	1,2,4	*B.paramycoides*	MW700024	+	-
	CTA46	1413	99.9	3,4,5	*Bacillus* sp. (*B. proteolyticus*/*B. wiedmannii*)	MW700027	+ *	+
	CTB34	1414	99.9	1,2,3,5	*B.cereus*	MW700048	-	-
	CTB116	1405	100	2,5	*B.cereus*	MW700056	-	-
F	CTA39	1349	99.9	3,4,5	*S. pasteuri*	MW700026	-	-
	CTA79	1370	99.9	1,5	*S.cohnii*	MW700029	-	-
	CTB46	1411	99.9	1,2,4	*S.cohnii*	MW700050	-	+
G	CTA107	897	100	2,5	*P. stutzeri*	MW700034	-	-
	CT110	1322	100	2,4	*A. lwoffii*	MW700055	-	-
H	CTA23	1375	99.8	2,4,5	*P. fulva*	MW700022	+ *	-
	CTA90	708	99.9	1,3	*Pseudomonas* sp. (*P. putida*/*P.fulva*/*P.parafulva*/ *P. guariconensis*/*P. plecoglossicida*/ *P. cremoricolorata*/*P. taiwanensis*/ *P. donghuensis*/*P.wadenswilerensis*/ *P. alkylphenolica*)	MW700031	+ *	+
I	CTA125	1436	98.9	1,2	*Bacillus* sp.	MW700036	-	+ *
J	CTA25	1371	99.9	2,4,5	*Terribacillus* sp. (*T. goriensis*/*T. saccharophylus*)	MW700023	-	-
K	CTA84	1278	99.8	2,3	*P.coleopterorum*	MW700030	-	-
L	CTA95	1333	99.9	1,3	*M. imperiale*	MW700032	+ *	-
M	CTA99	1349	100	1,3	*Bacillus* sp. (*B. proteolyticus*/*B. wiedmannii*)	MW700033	-	-
O	CTA138	1405	99.9	2,4	*Bacillus* sp. (*B. proteolyticus*/*B. wiedmannii*)	MW700037	-	-
	CTB60	1404	99.9	3,4,5	*L. fusiformis*	MW700051	-	-
S	CTA169	496	99.8	3,5	*P. profundus*	MW700040	+ *	+ *
T	CTB32	1382	99.9	1,2,3,5	*M. yunnanensis*	MW700047	-	-

Table 2. *Cont.*

Group	Strain	bp	% Identity	Antibacterial Activity [1]	Bacteria	Accession Number	NRPS	PKS
W	CTB93	1379	99.8	1,2	*T. saccharophilus*	MW700054	-	-
Y	CTB69	1416	99.6	2,4,5	*Paenibacillus* sp. (*P. cucumis*/*P. polysaccharolyticus*)	MW700052	+ *	+ *
LAB1	CT2H	440	99.8	1,2,3,4	*S. epidermidis*	MW700058	-	-
	CT7C	580	100	3,4,5	*S. warneri*	MW700060	-	-
LAB2	CT6C	563	99.6	1,2,3,4,5	*S.arlettae*	MW700059	+	-
LAB3	CT12B	432	99.8	1,2,5	*S.hominis*	MW700061	-	-

* Amplicons were sequenced. [1] (1) *S. aureus*, (2) *P. aeruginosa*, (3) *S.* Typhimurium, (4) *A. baumani*, (5) *C. freundii.*

Furthermore, all 50 isolates were screened for the presence of NRPS and PKS-I biosynthetic gene clusters. In fourteen isolates, NRPS genes were detected; six isolates were tested positive for PKS gene clusters whereas in 13 isolates both NRPS and PKS gene clusters were detected. Twenty-six out of 31 (83.9%) bacterial isolates identified as *Bacillus* spp. tested positive for NRPS or PKS or both. Specifically, 11 out of the 31 (35.5%) bacterial isolates identified as *Bacillus* spp. tested positive only for NRPS genes (78.6% of the isolates positive for NRPS, 11/14), 5 were positive only for PKS genes (83.3% of the isolates positive for PKS, 5/6) and 10 isolates were detected positive for both NRPS/PKS gene clusters (76.9% of all isolates positive for both NRPS and PKS, 10/13). Moreover, only NRPS genes were detected in 4 out of 5 identified *B. pumilus* strains (CTA1, CTA2, CTA15, CTB21), in 2 out of 9 *Bacillus* sp. (CTA31, CTA14), and in 2 out of 7 *B. safensis* strains (CTB16, CTB120). *B. licheniformis* (CTA163), *P. megaterium* (CTA7), and *B. paramycoides* (CTA28) were also positive just for NRPS genes. Two *B. safensis* (CTA146, CTB7) and 3 *Bacillus* sp. (CTA109, CTB11, CTA125) strains were positive just for PKS gene clusters. Both NRPS and PKS genes were detected in *B. vallismortis* (CTA16), in 4 *B. subtilis* strains (CTA5, CTA9, CTA10, CTA20), 3 *B. safensis* (CTA57, CTB43, CTB89), and 2 *Bacillus* spp. (CTB31, CTA46) strains.

Two out of 4 (50%) bacterial isolates identified as *Pseudomonas* spp. tested positive for NRPS (*P. fulva* CTA23) or both NRPS/PKS (*Pseudomonas* sp. CTA90) gene clusters. In addition, 2 out of 7 isolates identified as *Staphylococcus* spp. tested positive for NRPS (*S. arlettae* CT6C) and PKS (*S. cohnii* CTB46) gene clusters. The strain *M. imperiale* (CTA95) was positive for NRPS encoding genes, while in *P. profundus* (CTA169) and *Paenibacillus sp*. (CTB69) both NPRS and PKS gene clusters were detected.

Twelve NRPS amplicons and 11 PKS amplicons were sequenced, in order to validate the PCR result (Tables 3 and 4). Ten out of 12 amplicons were indeed identified as NRPS gene clusters. The identification of the other two amplicons was not possible due to low quality of sequencing data. In most cases there was an agreement regarding the genus classification and sequencing results, except for amplicons A1 and A7 that were amplified from bacterial isolates belonging to *Bacillus* spp., whereas the NPRS amplicons shared homology with NRPSs of *P. moteilii*. Amplicons A10, A20, and A46 from the strains *B. safensis* (CTA10), *B. safensis* (CTA20), and *Bacillus* sp. (CTA46) were identified as NRPSs that were implicated in surfactin biosynthesis, while B69 from the strain *Paenibacillus* sp. shared homology with the subunit B of gramicidin synthase.

Furthermore, the sequencing data verified the PCR results for the 11 tested PKS amplicons. Six amplicons exhibited homology with ketosynthases, (A5, A9, A10, A20, A125, B11), 3 were identified as PKSs (A109, B31, B69), while amplicon A16 was identified as amino acid adenylation domain-containing protein. In all cases there was an agreement regarding the classification at genus level and the sequencing results. Interestingly, sequencing data regarding A9 amplicon of *B. subtilis* CTA9 strain suggest that bacillaene is biosynthesized by this strain.

Table 3. Sequencing results for the NRPS amplicons.

Sample	Species	BLASTN	% Identity	BLASTX	% Identity
A1	*B. pumilus*	*P. monteilii* (AMA45849.1 hypothetical protein)	84.4	amino acid adenylation domain-containing protein (*P. monteilii*)	78.7
A7	*P. megaterium*	*P. monteilii* (Gene APT63_12835, AMA46431.1 hypothetical protein)	67.2	NRPS (*P. monteilii*)	41.7
A10	*B. subtilis*	*B. subtilis* strain JCL16 chromosome (Gene srfAA), QKJ76470.1 surfactin NRPS SrfAA	84.2	Surfactin NRPS SrfAA (*B. subtilis*)	72.7
A14	*Bacillus* sp.	*B. licheniformis* strain P8_B2 chromosome, QGI45530.1 D-alanine–poly(phosphoribitol) ligase subunit DltA	95.9	D-alanine–poly(phosphoribitol) ligase subunit DltA (*B. licheniformis*)	91.1
A20	*B. subtilis*	*B. subtilis* subsp. subtilis str. 168 chromosome (QJR44804.1 surfactin NRPS SrfAA	98.1	surfactin NRPS SrfAA (*B. subtilis*)	98.6
A23	*P. fulva*	*P. monteilii* strain USDA-ARS-USMARC-56711 (Gene APT63_09540, AMA45849.1, hypothetical protein	96.5	amino acid adenylation domain-containing protein (*P. monteilii*)	98.4
A46	*Bacillus* sp.	*B. safensis* strain PgKB20 chromosome Surfactin synthase subunit 2 (gene srfAB_2)	96.5	NRPS (*B. safensis*)	99.49
A90.2	*Pseudomonas* sp.	*P. monteilii* strain USDA-ARS-USMARC-56711, gene APT63_09540, hypothetical protein	91.8	amino acid adenylation domain-containing protein (*P. monteilii*)	87.7
A95	*M. imperiale*	*M. oleivorans* strain I46 chromosome DNA gyrase subunit A	68.1	no	no
A163	*B. licheniformis*	*B. haynesii* strain P19 chromosome	73.2	no	no
A169	*P. profundus*	*P. thiaminolyticus* strain NRRL B-4156 amino acid adenylation domain-containing protein FLT43_25335	71.8	NRPS/PKS (*P. apiarius*)	56.5
B69	*Paenibacillus* sp.	*P. polymyxa* E681 isolate type B chromosome Linear gramicidin synthase subunit B, lgrB_2	83.5	AMP-binding protein (*P. amylolyticus*)	96.6

Table 4. Sequencing results for the PKS amplicons.

Sample	Species	BLASTN	% Identity	BLASTX	% Identity
A5	*B. subtilis*	*B. subtilis* subsp. subtilis str. 168 chromosome amino acid adenylation domain-containing protein, HIR77_09320	81.8	ketosynthase, partial (*Bacillus* sp.)	82.7
A9	*B. subtilis*	*B. subtilis* subsp. subtilis strain UCMB5021 Malonyl CoA-acyl carrier protein PksN transacylase involved in nonribosomal synthesis of bacillaene, gene pksN	77.1	ketosynthase, partial (*Bacillus* sp.)	76.1
A10	*B. subtilis*	*B. subtilis* strain JCL16 chromosome, amino acid adenylation domain-containing protein, gene HR084_09185	78.9	ketosynthase, partial (*Bacillus* sp.)	78.2
A16	*B. vallismortis*	*B. amyloliquefaciens* strain R8-25 chromosome (or *valezensis*) amino acid adenylation domain-containing protein, gene HT132_11940	76.4	amino acid adenylation domain-containing protein (*B. amyloliquefaciens*)	93.2
A20	*B. subtilis*	*B. subtilis* subsp. subtilis str. 168 chromosome, amino acid adenylation domain-containing protein, gene HIR77_09320	85.7	ketosynthase, partial (*Bacillus* sp.)	87.86
A109	*Bacillus* sp.	*P. thiaminolyticus* strain NRRL B-4156 chromosome acyltransferase domain-containing protein, gene FLT43_06730	81.2	acyltransferase domain-containing protein (*P. apiaries*)	100
A125	*Bacillus* sp.	*B. subtilis* strain JCL16 chromosome amino acid adenylation domain-containing protein, gene HR084_09185	79.2	ketosynthase, partial (*Bacillus* sp.)	77.7
A169	*P. profundus*	*P. thiaminolyticus* strain NRRL B-4156 chromosome acyltransferase domain-containing protein, gene FLT43_06730	81.1	acyltransferase domain-containing protein (*P. apiarius*)	100
B11	*Bacillus* sp.	*B. halotolerans* strain KKD1 chromosome NRPS, gene HT135_09465	99	type I ketosynthase (*Bacillus* sp. LX-99)	97.4
B31	*Bacillus* sp.	*Bacillus* sp. AM1(2019) chromosome zinc-binding dehydrogenase, gene GNE05_12430	72.9	PKS (*B. velezensis*)	60
B69	*Paenibacillus* sp.	*Paenibacillus* sp. B37 PKS gene, PKS	94.2	PKS (*Paenibacillus* sp. B15)	98.5

4. Discussion

The antimicrobial activity exerted by honey is one of its most well-established bioactivities described in many studies [11]. Honey affects the growth and survival of microorganisms via multiple mechanisms [14,41]. Low pH, high sugar concentration, high osmolarity and the presence of antibacterial substances such as hydrogen peroxide and phytochemicals in honey provide a hostile environment for bacteria [42]. Consequently, honey is expected to contain a limited number and diversity of microorganisms [14]. Nevertheless, honey is not sterile since it is a natural product that is not processed [3,5,12,28]. Several studies have reported isolated microorganisms from honey samples produced in various geographical locations [4,7,12,18,43]. Interestingly, the number of microorganisms in honey ranged widely, from 0 to several thousand colony forming units (CFUs) per gram, depending on the sample and its freshness [8,9,18]. Sinacori et al. [18] reported low bacterial load in 33 out of 38 honey samples harvested in southern Italy. Similar results were reported by other researchers in Argentina [8], Morocco [15], Poland [12], Saudi Arabia [44], and Mexico [7]. Fernández et al. [27] studied the microbiological quality of honey from Argentina and their results were comparable to those reported in previous studies conducted in Argentina as well as other parts of the world [8,12,18,28,45]. Bacteria have also been isolated from stingless bee honey [4,23,42]. Moreover, there are few studies that have described the microbial communities of honey using culture-independent methods like next generation sequencing (NGS) [17,46–48].

Honey bacteria exerting antibacterial activity have been successfully isolated by several research groups [6,12,42,49–52]. There are also reports on the antibacterial activity exerted by various bacterial isolates from stingless bee (*Heterotrigona itama*) honey [34,42]. Lee et al. [28] screened more than two thousand bacterial strains isolated from six US domestic honeys and two manuka honeys against seven bacteria and one mold but identification of bacterial isolates was not conducted. Recently, Pajor et al. [12] investigated the antimicrobial potential of 163 bacteria isolated from honey samples produced in Poland.

A number of studies have demonstrated high antibacterial activity of Greek honeys against major nosocomial and foodborne pathogens [1,13,35,53]. However, this is the first study aiming to isolate and characterize bacteria from diverse types of Greek honeys exhibiting antibacterial potential. In total, 2014 bacterial isolates were screened against five important human and foodborne pathogens (*S. aureus*, *P. aeruginosa*, *S.* Typhimurium, *A. baumannii*, and *C. freundii*). Three of them belong to the "ESKAPE" pathogens group (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.). The World Health Organization (WHO) included ESKAPE in a list of pathogens for which new antimicrobial compounds are urgently needed. *A. baumannii* and *P. aeruginosa* are listed in the critical priority list of pathogens, whereas *S. aureus* (MRSA) is in the high priority group [54–56]. Furthermore, multidrug-resistant *C. freundii* is of major concern, often implicated in nosocomial infections [57,58]. This is the first study regarding the antibacterial activity of honey bacterial isolates against *C. freundii* and *A. baumanii*.

Moreover, in this study a higher number of bacterial isolates (2014) was screened against five pathogens, compared to relevant studies [4,42,51,52]. The most susceptible pathogen was *S. aureus*, followed by *C. freundii*, *P. aeruginosa*, and *A. baumannii*. In contrast, *S.* Typhimurium was the least affected tested pathogen. A possible explanation might be that Gram-negative bacteria possess an outer membrane which often contributes to resistance to a wide range of antibacterial factors (for instance, loss of porins located in outer membrane). In agreement with our study, Pajor et al. [12] reported that 20.3% of the honey isolated strains inhibited the growth of *S. aureus* ATCC 25923, while activity against *P. aeruginosa* ATCC 27857 was rarely observed.

In previous studies, aerobic spore-forming bacteria belonging to *Bacillus* spp. and related genera (*Brevibacillus*, *Lysinibacillus*, *Paenibacillus*) have been identified as the most common bacteria present in honey [5,59]. Furthermore, bacteria belonging to *Bacillus* genus have been often isolated from the honeybee gut [60–62]. Similarly, in this study, 62% of the identified isolates belonged to the genus *Bacillus*. Malika et al. [15] detected *Bacillus*

spores in most of the honey samples from Morocco, while Pomastowski et al. [3] reported that regardless of the geographical and botanical origin, the main bacteria in honeys were spore-forming *Bacillus* spp., which is in agreement with our data.

Bacteria belonging to *B. subtilis* or *B. cereus* groups are the most frequently detected in honey [3,25]. Indeed, *B. safensis* was the most frequently identified species in our study followed by *B. pumilus*, *B. subtilis*, and *B. cereus*. The species *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. safensis*, *B. subtilis*, and *B. vallismortis* have been detected in honey samples from diverse geographical locations [3,4,9,22,25,42,44,60,63].

Nevertheless, to the best of our knowledge, this is the first time that *B. paramycoides*, *L. fusiformis*, *M. imperiale*, *M. yunnanensis*, *P. profundus*, and *T. saccharophilus* were isolated from honey. *Paenibacillus* strains isolated from various ecological niches synthesize a wide range of antibiotics (polymyxins, polypeptins, gavaserin) [51,64]. *P. profundus* strain Sl 79, a deep sediment bacterium, exerted a remarkable inhibitory activity against both Gram-positive and Gram-negative bacteria. It is known to produce isocoumarin and a new linear glyceryl acid-derived heptapeptide that exhibited antibacterial activity against *S. aureus*, *S. epidermidis*, *B. subtilis*, and *E. faecium* [64,65]. On the other hand, *M. yunnanensis* was previously detected in bee gut [61,66] but not in honey. Micrococcin was the first thiazolyl peptide isolated from a *Micrococcus* strain [67]. Sponge-derived *M. yunnanensis* F-256,44 was reported to produce kocurin, an antibiotic of the thiazolyl peptide family [67,68]. Moreover, Ravi Ranjan et al. [69] isolated *M. yunnanensis* strain rsk5, which produced a novel antibacterial compound. This compound exerted activity against antibiotic-resistant *S. aureus* and differs from other characterized secondary metabolites. *T. saccharophilus*, as its name suggests, is a sugar-loving bacterium [70]. A study reported that a *T. saccharophillus* strain isolated from the gut of an edible Indian freshwater fish could be applied as a probiotic in fish feed formulations [71]. Another study reported the production of copious amounts of exopolysaccharides by *T. saccharophilus* strain PS-47. Exopolysaccharides are secondary metabolites, used in the pharmaceutical and food industries [72]. *Microbacterium* spp. were previously isolated from the gastrointestinal tract of adult worker honeybees [62]. A study reported that *M. imperiale* (MAIIF2a) reduced fungal symptoms by *Fusarium solani* in cassava roots, indicating its antimicrobial potential [73].

The antibacterial activity of *B. paramycoides* and *L. fusiformis* against important human and food pathogens has been described for the first time in this study. However, these bacterial species are known for their bioremediation potential [74–76]. Moreover, *B. paramycoides* was reported as a plant growth-promoting rhizobacterium [77], while *L. fusiformis* strain C250R produced a novel extracellular thermostable protease designated as SAPLF, which could be applied in pharmaceuticals, food, and biotechnology industries [78].

In this study, 14% of the characterized bacterial isolates were identified as *Staphylococcus* spp. Previous studies have reported the presence of *S. epidermidis*, *S. pasteuri*, and *S. hominis* in honey [3,22,79]. Moreover, all *Staphylococcus* spp. identified in this study, except *S. arlettae*, have been described as part of the honeybee gastrointestinal microbiome [3,61,62,66,80,81]. *S. arlettae* is commonly found in the skin and nares of poultry and goats [82].

Surprisingly, none of the bacterial isolates grown on MRS agar that exerted antimicrobial acitivity in this study was identified as lactic acid bacteria. However, all four isolates were identified as *Staphylococcus* spp., one of them (*S. hominis*) previously isolated using MRS agar [83].

Only 10% of the characterized bacterial isolates were identified as Gram-negative bacteria, namely *Pseudomonas* sp., *P. coleopterorum*, *P. fulva*, *P. stutzeri*, and *A. lwoffii*. *Pseudomonas* spp. and *Actinetobacter* spp. are often present in soil [7]. However, Veress et al. [84] isolated for the first time the bacterium *A. lwoffii* from a Transylvanian honey sample, suggesting that it was derived from the honeybee microbiota. *Pseudomonas* spp. have been identified both in honey and the digestive tract of honeybees [9,10,17,22,62]. The *Pseudomonas* genus is very diverse and comprises 272 species, organized in several major taxonomic groups that are widely distributed in diverse niches [85]. Some of these strains

exert biotechnologically interesting metabolic potential (denitrification, degradation of aromatic compounds, nitrogen fixation) [86–89]. *P. fulva* is a widespread environmental species exhibiting high biodegrading potential. It is reported to degrade neonicotinoid insecticides that contribute to honeybee colony collapse disorder [86], as well as a pyrethroid insecticide, reported to be accumulated in honeybee wax samples [87]. On the other hand, *P. coleopterorum* was detected in the gut microbiota of some plant-related insects [90].

Bee products have been suggested as a rich source of beneficial bacteria that might be potential biocontrol agents against bee pathogens [4,28,42]. Several *Bacillus* strains produce compounds against pathogenic and food-spoilage bacteria [2,51]. Moreover, *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. licheniformis* are considered as probiotics and antibiotic producers. Indeed, *B. subtilis* has already been used as a probiotic supplement for human and animal diets [23,42].

LABs commonly isolated from honey produce secondary metabolites, like bacteriocins and lipopeptides [6,42,91,92]. However, we did not identify any LABs exhibiting antibacterial activity in the present study, presumably due to the relatively small number of isolates grown on MRS agar.

Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are often implicated in the biosynthesis of medically useful secondary metabolites, such as antibiotics, antitumor agents, immunosuppressants, siderophores, and toxins [30,31,33]. In this study we report the detection of genes encoding NRPSs and PKSs in 14 and 6 out of 50 identified isolates, respectively. NRPS genes were detected more often in *Bacillus* spp. It is known that *Bacillus* and *Paenibacillus* genera produce non-ribosomally synthesized lipopetides with significant antimicrobial activity, such as iturin, fengycin, surfactin, and bacillomycin. These compounds are abundantly synthesized, particularly by *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. pumilus* [92,93]. Furthermore, NRPs have been identified in *Pseudomonas* spp. by several studies [94–97]. Analysis of *P. fluorescens* In5 revealed the presence of two antifungal NRPs, nunamycin and nunapeptin [94]. Another study showed that a monomodular NRPS from *Pseudomonas fluorescens* HKI0770 is implicated in biosynthesis of the antimicrobial pyreudiones [96].

Magdalena et al. [51] have targeted seven antibacterial peptide biosynthetic genes present in two *B. velezensis* honey isolates using specific primers. However, in our study the universal primer set NRPS-1F—NRPS-1R for the amplification of adenylation (A) domain was used, thus targeting a much broader range of NRPS biosynthetic clusters.

PKS gene clusters were detected in *B. safensis*, *Bacillus* sp., and *S. cohnii* strains. Type I PKSs are large multifunctional enzymes, implicated in pharmacologically important metabolites, most often detected within prokaryotes [93,98]. PKs were detected in *B. subtilis*, *B. amyloliquefaciens*, *B. methylotrophicus*, *B. atrophaeus*, *B. laterosporus*, and *Paenibacillus* sp. [93]. Within the *B. subtilis* group, three types of antimicrobial PKs are produced, including bacillaene, difficidin, and macrolactin [98]. Moreover, Zhang et al. [99], reported the partial isolation of a gene cluster from *Staphylococcus lentus* encoding a PK exerting broad-spectrum activity against fungi and bacteria. To the best of our knowledge, this is the first time that PKS encoding genes have been detected in bacteria isolated from honey exerting antimicrobial potential.

Interestingly, 13 isolates in this study contained both NRPS and PKS gene clusters. Often NRPSs and PKSs are synthesized as hybrid products by NRPS and PKS gene clusters [31,33,100], such as bacillaene, compactin, fusarin C, or salinosporamide [98].

Twelve NRPS amplicons and 11 PKS amplicons were sequenced, in order to validate our detection approach. Three out of the 12 NRPS amplicons were implicated in the synthesis of surfactin. Surfactin is a cyclic lipoheptapeptide produced by various strains of *Bacillus subtilis*, which alters membrane integrity and permeability acting against several microorganisms [51,92,101].

Furthermore, it has been reported that *Paenibacillus* strains, isolated from various environments, produce a wide range of antibiotics [64,92]. Three *Paenibacillus* species were detected in honey, *P. alvei*, *P. polymyxa*, and *P. larvae* [3,28,92,102]. Polymyxins produced by

P. polymyxa have been used as a last-resort treatment of multidrug-resistant Gram-negative bacteria infections. They also exhibit strong antifungal activity [93].

Finally, our sequencing results regarding the PKS amplicon from *Bacillus subtilis* CTA9 strain suggest the presence of a PKS implicated in bacillaene biosynthesis. Bacillaene was firstly isolated from *B. subtilis* strains, but since then has been reported to be produced by several other *Bacillus* spp. It is a hybrid PK synthesized by a PKS-I and NRPS operon and it has demonstrated a strong activity against various bacteria and fungi [92,93,98]. Taking together all these data, it is evident that most of the bacterial isolates in this study could be potent producers of secondary metabolites including antibiotics, bacteriocins, and enzymes that might lead to future biotechnological applications.

5. Conclusions

In the present study, 2014 bacterial isolates were obtained from 46 Greek honey samples of diverse botanical and geographical origin. All bacterial isolates were tested against five important nosocomial and foodborne pathogens. Overall, 31.5% of the isolates exerted antibacterial activity against at least one pathogen. Gram-positive *S. aureus* was the most susceptible of the five tested pathogens, followed by *C. freundii*, *P. aeruginosa*, and *A. baumanii*. *S.* Typhimurium was found to be the least susceptible pathogenic bacterium. Fifty isolates that inhibited the growth of at least two tested pathogens were further characterized. Most of them (62%) were identified as *Bacillus* spp. Nevertheless, six identified species were isolated from honey samples for the very first time. Moreover, the detection of NRPS and PKS (PKS-I) biosynthetic gene clusters in several isolates is reported, underlining the high biosynthetic potential of honey bacteria. Collectively, the results presented in this study demonstrate that the honey microbiome is an untapped source of antimicrobial metabolites against multidrug-resistant nosocomial and food pathogens that warrants further investigation and bioprospecting.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11135801/s1, Table S1: Honey samples of diverse botanical, geographical origin, and harvest date. Number of bacterial isolates per honey sample and per culture medium, Table S2: Grouping of bacterial isolates by RFLP assay and Gram staining.

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