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Metabolomic Profiling and Biological Activities of Cell-Free Supernatants of *Bacillus* sp. Isolates: Antibacterial, Antibiofilm, and Anti-quorum Sensing Activities

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ABSTRACT

Objective: The objective of this research was to explore the antibacterial, antibiofilm, and anti-quorum sensing (anti-QS) activities of cell-free supernatants (CFSs) of *Bacillus* sp. and correlate these activities with their metabolite profiles.

Materials and Methods: We used 55 *Bacillus* sp. isolates from soil samples. The antibacterial activities of the CFSs were investigated using disk diffusion and agar well diffusion methods. Antibiofilm activities were assessed using the crystal violet microplate method and anti-QS activities were evaluated using the disc diffusion method. The identification of biologically active isolates was performed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS). The metabolite profiles of active CFSs were analyzed using gas chromatography-mass spectrometry (GC-MS).

Results: Only S-45 exhibited antibacterial activity against *Staphylococcus aureus* ATCC 25923. Biofilm inhibition percentages of the CFSs against *Staphylococcus epidermidis* ATCC 35984 and *Pseudomonas aeruginosa* PAO1 varied between 92.58% - 3.96% and 78.96% - 0.64%, respectively. Only S-37 exhibited anti-QS activity. The most biologically active isolates belonged to the *Bacillus cereus* group. Based on the GC-MS results, 102 metabolites were identified. According to correlation analyses between the results of antibiofilm activity and metabolite profiles, we determined that compounds belonging to the amino acid and peptide groups, hydroxy acids and derivatives, and fatty acyls exhibited a highly positive correlation. The metabolite profiles of S-45 and S-37 were not significantly different from the negative control (S-46). Therefore, these activities could not be associated with the metabolite content.

Conclusion: The correlation between specific metabolite profiles and bioactivities indicates the potential of these bioactivities for innovative pharmaceutical applications. Future research should focus on isolating and testing these individual metabolites to confirm their specific roles and mechanisms.

Keywords: Antibacterial activity, antibiofilm activity, anti-quorum sensing activity, *Bacillus* sp., GC-MS, metabolomics

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INTRODUCTION

Antibiotic resistance is considered one of the most critical threats to humanity's future (1). Bacterial infections caused by resistant strains lead to prolonged hospitalization, increased healthcare costs, treatment failures, and mortality (2, 3). Safe and effective novel compounds that can act through different mechanisms are needed to fight antibiotic resistance (4).

The inadequacy of existing antibacterials for treating resistant bacterial infections and the inability to discover new antibacterials have directed researchers to investigate molecules that can inhibit the mechanisms associated with pathogenicity (5). Bacteria communicate with each other to synthesize virulence factors that contribute to their pathogenicity. This communication mechanism, achieved through extracellular chemical signaling molecules, is referred to as quorum sensing (QS). Bacterial processes such as bioluminescence, conjugation, biofilm production, and sporulation are known to be regulated by the QS mechanism (6). A biofilm is a collection of microbial cells attached to a surface and surrounded by extracellular polymeric substances. Biofilm formation is a mechanism by which bacteria maintain their viability (7).

Plants, bacteria, marine organisms, terrestrial vertebrates, and invertebrates are sources of active natural compounds that can be used in medicine (8). Metabolites produced by many microorganisms in different natural habitats are widely used in fields such as medicine, pharmacy, agriculture, and biotechnology (9). Soil is an environment that exhibits a diversity of organic and inorganic matter. Soil properties and environmental conditions influence the composition of microorganisms and their metabolite content (10,11). The genus *Bacillus* is a predominant group of bacteria in soil microflora (12). The members of the genus *Bacillus* are industrially important due to their easy production, resistance to adverse environmental conditions, and metabolic properties such as antibiotic, enzyme, organic acid, pigment, and toxin production. Bacitracin, subtilin, ricin, mersacidin, amicoumacin, subtilosin produced by *Bacillus subtilis (B. subtilis),* polymyxin produced by *paenibacillus polymyxa*, pumilio pumilin produced by *B. pumilus*, and iturins produced by *B. subtilis* and *B. amyloliquefaciens* can be given as examples of antibiotics. However, subtilisin produced by *B. subtilis* and amylase produced by *B. amyloliquefaciens* are examples of enzymes. Surfactin, fengycin, and iturin from *B. subtilis* are examples of surfactants, antifungals, and antivirals, respectively (10,13-15). Metabolites produced by *Bacillus* species, such as surfactants, alpha-amylase, and bacteriocin, have been shown to exhibit antibiofilm effects by disrupting and inhibiting the formation of bacterial biofilms (16). Metabolites from *Bacillus* species, such as lactonase enzyme and fengycin, have been shown to exhibit anti-QS effects by interfering with bacterial QS systems (17-19).

The purpose of this study was to investigate the antibacterial, antibiofilm, and anti-QS activities of *Bacillus* sp. cell-free supernatants (CFSs) and correlated these activities with their metabolite profiles. The findings of this study are significant because the antibacterial, antibiofilm, and anti-QS activities exhibited by the metabolites produced by *Bacillus* sp. suggest promising alternatives for combating antibiotic-resistant bacterial infections. Furthermore, the correlation between specific metabolite profiles and their bioactivities indicates considerable potential for innovative pharmaceutical applications.

MATERIALS AND METHODS

Isolation and Cultivation of Bacterial Strains

In this study, 74 soil samples were collected from various regions of Ankara, Afyonkarahisar, and Istanbul. In order to characterize the soil samples used in this study, detailed information regarding the regions from which the samples were collected, including their specific properties, is provided in Table 1. Each soil sample (approximately 10 gr in weight) was collected using clean, dry, and sterile amber-colored glass bottles, along with a sterile spatula. To prepare the samples for isolation, 1 gr of each soil sample was mixed with 10 mL of sterile distilled water. The suspension was then heated to 100°C and maintained at 100°C for 5 min to eliminate vegetative cells. Serial dilutions (10^{-1} to 10^{-4}) were prepared from the samples, and 0.1 mL of each dilution was spread onto Nutrient Agar (NA) (Merck, Germany) plates. The plates were incubated at 37°C for 18-24 h. Following incubation, the selected colonies were stained using Gram staining. Gram-positive and rod-shaped bacilli were selected for further identification tests, which included assessments of spore-forming capability, motility, and catalase activity (20).

Identification of Bacterial Species

The identification of biologically active isolates was performed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) (Bio Mérieux, France). Bacterial cultures were incubated on blood agar plates at 37°C for 24 h. A single bacterial colony was picked using a 1 µL sterile loop and applied to a metal target plate. Subsequently, 1 µL of matrix solution (α-cyano-4-hydroxycinnamic acid; CHCA) was added to the sample on the target plate. The prepared target plate was then loaded into the device for analysis. The spectral data obtained from the MALDI-TOF-MS were compared against the reference library to identify the bacterial isolates (21).

Obtaining Cell-Free Supernatants

To obtain the CFSs from the *Bacillus* sp. isolates, cultures from 24 h NA plates were inoculated into Luria Bertani Broth (LBB) (Merck, Germany) and incubated at 37°C for 72 h. To eliminate the bacterial cells from the medium, the incubated samples were centrifuged at 12.000 g for 10 min at 4°C. Next, the supernatants were filtered through a 0.45-µm pore filter (20).

Table 1. Regions where soil samples were collected and their properties

Soil Sample	Region	Collection Date	Soil Sample	Region	Collection Date	Soil Sample	Region	Collection Date
S-1	*Platanus orientalis* tree, Ankara	March 2021	**S-20**	Rocky ground, Ankara	May 2021	**S-39**	*Pinus sp.* tree, Ankara	August 2021
S-2	Next to the manhole cover, Ankara	March 2021	**S-21**	İmrahor Valley, Ankara	May 2021	**S-40**	*Pinus sp.* tree, Ankara	August 2021
S-3	Next to the pool drain, Ankara	March 2021	**S-22**	Imrahor Valley, Ankara	May 2021	**S-41**	*Pinus sp.* tree, Ankara	August 2021
S-4	*Aesculus hippocastanum* tree, Ankara	March 2021	**S-23**	*Salix alba* L. Ankara	May 2021	**S-42**	Potted, Ankara	September 2021
S-5	Next to the manhole cover, Ankara	March 2021	**S-24**	*Salix alba* L. Ankara	May 2021	**S-43**	Potted, Ankara	September 2021
S-6	Thermal water, Afyonkarahisar	April 2021	**S-25**	*Pinus pinea* tree, Ankara	July 2021	**S-44**	Shrub soil, Ankara	September 2021
S-7	Garden soil, Afyonkarahisar	April 2021	**S-26**	*Eymir Lake,* Ankara	July 2021	**S-45**	Near the water canal, İstanbul	August 2021
S-8	Cemetery garden, Afyonkarahisar	April 2021	**S-27**	*Eymir Lake,* Ankara	July 2021	**S-46**	Garden soil, Ankara	September 2021
S-9	Garden soil, Afyonkarahisar	April 2021	**S-28**	*Eymir Lake,* Ankara	July 2021	**S-47**	*Pinus sp.* tree, Ankara	September 2021
S-10	*Fraxinus excelsior* tree, Afyonkarahisar	April 2021	**S-29**	*Eymir Lake,* Ankara	July 2021	**S-48**	Shrub soil, Ankara	September 2021
S-11	*Rose* sp., Ankara	April 2021	**S-30**	*Eymir Lake,* Ankara	July 2021	**S-49**	Potted plant, Ankara	September 2021
S-12	*Rose* sp., Ankara	April 2021	**S-31**	*Eymir Lake,* Ankara	July 2021	**S-50**	Garden soil, Ankara	September 2021
S-13	*Pinus pinea* tree, Ankara	April 2021	**S-32**	Shrub soil, Ankara	July 2021	**S-51**	Riverbed, Ankara	September 2021
S-14	*Pinus pinea* tree, Ankara	April 2021	**S-33**	Shrub soil, Ankara	July 2021	**S-52**	Riverbed, Ankara	September 2021
S-15	*Pinus pinea* tree, Ankara	April 2021	**S-34**	Shrub soil, Ankara	August 2021	**S-53**	Riverbed, Ankara	September 2021
S-16	*Pinus pinea* tree, Ankara	April 2021	**S-35**	Landscape plants, Ankara	August 2021	**S-54**	Riverbed, Ankara	September 2021
S-17	Potted Ankara	April 2021	**S-36**	*Pinus pinea* tree, Ankara	August 2021	**S-55**	Landscape plants, Ankara	September 2021
S-18	Potted Ankara	April 2021	**S-37**	Garden soil, Ankara	August 2021			
S-19	Potted Ankara	April 2021	**S-38**	*Prunus cerasifera* tree, Ankara	August 2021			

Table 2. The bacteria identified by MALDI-TOF-MS

Observed Activity	Isolate	MALDI-TOF-MS
Antibacterial activity	45	*B. cereus*
Anti-QS activity	37	*B. cereus*
Antibiofilm activity	21	*B. cereus*
	14	*B. cereus*
	16	*B. cereus*
	17	*B. altitudinis/pumilus*
	23	*B. subtilis/amyloliquefaciens/ vallismortis*
	22	*B. altitudinis/pumilus*
	15	*Priestia megaterium (B. megaterium)*
	18	*Peribacillus simplex (B. simplex)*
	19	*B. cereus*
	24	*B. cereus*
	12	*B. cereus*
	20	*Peribacillus simplex (B. simplex)*
	25	*B. cereus*
	43	*Peribacillus simplex (B. simplex)*
	36	*B. subtilis/amyloliquefaciens/ vallismortis*
	34	*B. cereus*

Antibacterial Activity Tests

To assess the antibacterial activities of the CFSs, agar well diffusion and disk diffusion methods were used. The two techniques were used together to evaluate the antibacterial activity more comprehensively. The tested bacterial strains were *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 1709, *Escherichia coli (E. coli) ATCC 25922, Pseudomonas aeruginosa (P. aeruginosa) ATCC 27853,* and *Staphylococcus aureus (S. aureus)* ATCC 25923.

Bacterial cultures grown for 18-24 h were adjusted to McFarland 0.5 and then spread onto Mueller-Hinton Agar (MHA) (Merck, Germany) plates. In the disk diffusion method, the CFSs were impregnated into sterile blank discs with a diameter of 6 mm (20 µL). After placing the impregnated disks on the inoculated media, the plates were incubated at 35 ± 1°C for 18-20 hours. Ciprofloxacin (5 µg) was used as the positive control, and Mueller-Hinton Broth (MHB) (Merck, Germany) was used as the negative control (19, 22, 23). For the agar well diffusion method, four wells (6 mm diameter) were made in plates containing the media inoculated standard test bacteria. Subsequently, 20 µL of sterile MHA, cooled to 45°C, was added to the bottom of each well. Next, 50 µL of CFS was added to each well. Incubation of the plates was carried out at 35 ± 1°C for 18-20 h. After this period, the diameters of the inhibition zones were measured (23).

Antibiofilm Activity Test

The antibiofilm activities of the CFSs were investigated using the crystal violet microplate method with *P. aeruginosa* PAO1 and *S. epidermidis* ATCC 35984 as test bacteria (24, 25).

The test bacteria were cultivated in Brain Heart Infusion (BHI) broth (Merck, Germany). Following incubation, the inoculum was adjusted to 10^6 cfu/mL with BHI broth supplemented with 2% sucrose. Subsequently, 100 µL of the bacterial suspension was added to each well of 96-well plates. The plates were incubated at 37°C for 24 h to allow biofilm formation. After the incubation period, the medium was removed, and non-adherent bacterial cells were washed away with sterile phosphate-buffered saline (PBS, pH 7.2).

Next, 100 µL of CFSs were added to the wells containing biofilms and incubated again at 37°C for 24 h. After incubation, the well contents were discarded, and the wells were rinsed with PBS. The plates were then allowed to dry at room temperature for an hour. Subsequently, 100 µL of a 0.5% crystal violet solution was transferred to each well to stain the biofilm cells. Following 30 min, the wells were washed using PBS. Next, the wells were treated with an acetone-alcohol (30:70, v/v) solution to dissolve the dye within the biofilm matrix. For the negative control, BHI broth containing 2% sucrose was used.

The optical density of the dissolved crystal violet at 620 nm (OD 620 nm) was measured using a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Vantaa, Finland). The percentage of biofilm inhibition values was calculated using the following formula:

Biofilm inhibition (%) = [(OD (control) – OD (sample))/OD (control)] x 100

Anti-Quorum Sensing Activity

The anti-QS activities of the CFSs were assessed using the disc diffusion method with the reporter bacteria *Chromobacterium violaceum* ATCC 12472 (25, 26).

The test bacterium was cultured in LBB, and the density of the overnight bacterial suspension was adjusted to 1.5×10^8 CFU/mL. After spreading the bacterial suspension onto Luria Bertani Agar (LBA) (Merck, Germany), sterile blank discs (Bioanalyse®, Ankara, Turkiye) (6 mm diameter) impregnated with 20 µL of the CFSs were placed. Following a 24-h incubation period at 30°C, the plates were examined for the presence of a violacein inhibition zone. The formation of an inhibition zone around the disks indicates potential anti-QS activity.

Table 3. GC/MS based metabolomic profiling (Major metabolites)

Super Class	Class /Subclass/ Parent	Number of Metabolites
Organic acids and derivatives	Amino acids, peptides, and analogs	32
	Hydroxy acids and derivatives	5
	Dicarboxylic acids and derivatives	4
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	22
Lipids and lipid-like molecules	Fatty acyls	4
	Glycerolipids	3
Benzeneoids	Benzene and substituted derivatives	1
	Phenols	1
Organoheterocyclic compounds	Indoles, and derivatives, diazines, dihydrofurans, lactones, pyridines and derivatives	10
Phenylpropanoids and polyketides	Cinnamic acids and derivatives, phenylpropanoic acids, coumarins and derivatives	4

GC-MS Metabolomics Analysis of CFSs

The metabolite profiles of the CFSs were analyzed using GC-MS-based metabolomics, as previously described (27). A GC-MS (Shimadzu GCMS-QP2010 Ultra) with a DB-5MS stationary phase column (30 m + 10 m DuraGuard × 0.25 mm i.d. and 0.25-µm film thickness) was used for metabolite analysis. The oven temperature program was set to 60°C for 1 min, increased to 325°C, at a 10 minute ramp and maintained for 10 min at 325°C. This resulted in a total separation time of 37.5 min. The MS detector was used in electron ionization mode (EI). Data acquisition was performed in full-scan mode. MS-DIAL software version 3.96 was used for peak detection, alignment, and deconvolution. In the pre-data process, a minimum peak height of 1000 amplitude was selected, and the other parameters were set to their default values. The identification cutoff value was set at 70%, and the retention time tolerance was 1 min. The Fiehn retention index database was used for metabolite identification.

Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to condense multidimensional data into fewer dimensions while keeping the majority of the original data. In this study, PCA was used to visualize the distribution of the isolates based on metabolite variations. MetaboAnalyst was used to create the PCA score plots (28).

Correlation Analysis

Microsoft Excel was used for the correlation analysis. The correlation function was used to determine the relationship between two properties (amounts of metabolites and activity results). The correlation coefficient reflects the level of correlation. As the correlation coefficient approaches +1 or -1, it indicates a positive (+1) or negative (-1) correlation between the properties. A positive correlation suggests that activity increases with increasing concentration. A coefficient closer to 0 denotes no or weak correlation.

RESULTS

Isolated/Identified *Bacillus* sp.

As a result of the identification tests, 55 *Bacillus* sp. were isolated from 74 diverse soil samples. The biological activity studies were conducted using the CFSs of these isolates. The identification of biologically active isolates was performed using MALDI-TOF-MS. The identified bacteria are listed in Table 2.

Antibacterial Activity of Cell-Free Supernatants

Based on the test results of 55 *Bacillus* sp. isolates, using the agar disk diffusion and agar well diffusion methods, only S-45 exhibited an inhibitory effect against *S. aureus* ATCC 25923 (Figure 1). None of the other CFSs used in this study demonstrated antibacterial activity against the test bacteria using either of the methods.

Antibiofilm Activity Test

The percentage of biofilm inhibition by CFSs against *S. epidermidis* ATCC 35984 varied between 92.58% and 3.96%. However, S-5, S-7, S-10, S-26, S-29, S-32, S-33, S-35, S-36, S-38, S-39, S-41, and S-46 did not exhibit any activity (Figure 2). For *P. aeruginosa* PAO1, the biofilm inhibition percentages of the CFSs varied between 78.96% and 0.64%. However, S-1, S-28, S-31, S-46, S-47, S-48, S-49, S-50, S-51, S-53, and S-54 showed no activity (Figure 3).

Table 4. Correlation analysis between the antibiofilm activity of *P. aeruginosa* PA01 and metabolite profiles ($r^2 \geq 0.70$ / $r^2 \leq -0.70$)

Correlation coefficient (r^2)	Metabolite name	Ontology
0.97	D-(+)-Malic acid	Beta hydroxy acids and derivatives
0.93	Homocystine	Alpha amino acids
0.87	Hexadecylglycerol	Monoalkylglycerols
0.84	N-Acetyl-L-Leucine	Leucine and derivatives
0.82	DOPA	Tyrosine and derivatives
0.79	Alanylalanine	Dipeptides
0.77	2-Deoxyguanosine	Purine 2'-deoxyribonucleosides
0.76	Glycolic acid	Alpha-hydroxy acids and derivatives
0.76	Putrescine	Monoalkylamines
0.76	Alanine-alanine	Dipeptides
0.76	n-Butylamine	Monoalkylamines
0.75	L-Ascorbic acid	Butenolides
0.75	Malonic acid	Dicarboxylic acids and derivatives
0.73	Saccharopine	Glutamic acid and derivatives
0.72	Thymine	Hydroxypyrimidines
0.70	N-Acetyl-L-aspartic acid	Aspartic acid and derivatives
-0.70	Nicotianamine	L-alpha-amino acids
-0.71	Spermidine	Dialkylamines
-0.72	Panose	Oligosaccharides
-0.77	Octacosanoic acid	Very long-chain fatty acids
-0.80	Lactitol	Fatty acyl glycosides of mono- and disaccharides
-0.81	Oxalic acid	Dicarboxylic acids and derivatives

Anti-Quorum Sensing Activity Test

A transparent inhibition zone around the disk indicates potential QS inhibitory activity. Among the samples, only S-37 showed a weak inhibition zone (Figure 4). In addition, none of the tested CFSs showed anti-QS activity.

GC-MS Metabolomics Analysis of CFSs

GC-MS metabolite profiling was performed to elucidate the compounds responsible for the observed activities of CFS numbers S-45 with antibacterial activity, S-37 with anti-QS activity, and S-16 and S-43 with high biofilm inhibition percentages. S-46, which showed no activity, was selected as the negative control in the study. In total, 102 primary and secondary metabolites were identified, and the class of the metabolites were summarized in Table 3.

The metabolite profiles of S-45 and S-37 did not differ significantly from the negative control. Therefore, these activities could not be associated with the metabolite contents of the samples.

Correlation Analysis

Correlation analyses were performed between CFSs with activity and metabolite profiles, and correlation coefficients (r^2) for each metabolite were calculated. As a result of the correlation analysis between the results of antibiofilm activity on *P. aeruginosa* PA01 and metabolite profiles, 16 metabolites exhibited a positive correlation ($r^2 \geq 0.70$), while 6 metabolites exhibited a negative correlation ($r^2 \leq -0.70$) (Table 4).

The correlation analysis between the results of antibiofilm activity on *S. epidermidis* ATCC 35984 and metabolite profiles showed that 9 metabolites have a positive correlation ($r^2 \geq$

Table 5. Correlation analysis between the antibiofilm activity of *S. epidermidis* ATCC 35984 and metabolite profiles ($r^2 \geq 0.70$ / $r^2 \leq -0.70$)

Correlation coefficient (r^2)	Metabolite name	Ontology
0.95	L-Asparagine	Asparagine and derivatives
0.85	L-Iditol	Sugar alcohols
0.85	N-Acetyl-DL-serine	N-acyl-L-alpha-amino acids
0.85	Urea	Non-metal pyrophosphates
0.83	L-Prolinamide	5-piperazinylimidazo[1,2-a] pyridines
0.78	N-Acetyl-L-Leucine	Leucine and derivatives
0.74	Glucose	Hexoses
0.74	Homocystine	Alpha amino acids
0.70	L-Norleucine	L-alpha-amino acids
-0.72	D-(+)-Mannose	Hexoses
-0.74	N-carbamoyl-L-aspartic acid	Aspartic acid and derivatives
-0.76	Hexose	Hexoses
-0.78	Oxalic acid	Dicarboxylic acids and derivatives
-0.90	Maltose	O-glycosyl compounds
-1.00	Spermine	Dialkylamines

0.70), while 6 metabolites have a negative correlation ($r^2 \leq$ -0.70) (Table 5).

PCA

A 3D PCA graph showing the similarity of the metabolite contents of the CFSs to each other is given below (Figure 5).

As seen in the PCA graphs, S-46's metabolite content was significantly different from the others. S-46 did not show any effect in the activity assays performed within the scope of our study. The other CFSs had similar metabolite profiles and gave positive results in activity assays.

DISCUSSION

Chemical compounds derived from various bacterial groups can have biological effects on pathogenic microorganisms. The metabolite content of soil bacteria may vary depending on factors such as the content of organic and inorganic matter, water ratio, and physical environmental conditions of soil samples (29).

Boottanun et al. reported that CFSs of *B. amyloliquefaciens* isolated from soil exhibited antibacterial effects against *S. aureus*, *Clostridium difficile*, *Enterococcus faecium*, *Burkholderia pseudomallei*, and *Acinetobacter baumannii* (20). Similarly, Thapa et al. investigated the antibacterial activity of secondary metabolites from various soil-derived *Bacillus* spp. using the agar well diffusion method against clinically significant pathogens, including *S. aureus*, *E. coli*, *Pseudomonas* spp., and *Salmonella* spp. (30). Their findings indicated that three out of the 24 isolates showed antibacterial properties against *S. aureus*, and three isolates (including one effective against *S. aureus*) were effective against *Salmonella* spp. Consistent with these findings, our study determined that the CFS from one isolate (S-45) exhibited a significant antibacterial effect against the Gram-positive bacterium *S. aureus* ATCC 25923.

QS is involved in the formation of various virulence factors of bacteria, including biofilm. It is considered a different strategy for the management of bacterial infections. El Aichar et al. investigated various biological activities of *Bacillus* strains isolated from soil and other ecological niches. The isolated *B. subtilis* DZ17 and *B. thuringiensis* DZ16 strains showed anti-QS and antibiofilm activities against *Streptococcus mutans* ATCC 25175 and *P. aeruginosa* PAO1 strains by inhibiting AI-2 and AHL signaling molecules (19). In a study conducted with *the C. violaceum* ATCC 12472 biosensor strain against *Serratia marcescens*, a causative agent of urinary tract infections, it was reported that *B. subtilis* R-18 extract showed anti-QS effect and inhibited bacterial adhesion in the early stages of biofilm formation (18). In our study, S-37 was found to inhibit the AHL bacterial communication molecule.

Nalini et al. investigated the antibiofilm activity of lipopeptide-producing *B. cereus* against the *E. coli* MTCC 2939 and *P. aeruginosa* MTCC 2453 strains. They reported biofilm inhibition rates of 56% and 62% for the lipopeptide-containing filtrates, respectively. In our study, using *P. aeruginosa* PAO1 as the test bacterium, the highest biofilm inhibition rate was observed for isolate S-43, achieving a value of 78.96% (31).

In our study, GC-MS analysis revealed that CFSs contain compounds from amino acid and peptides, cinnamic acids, alpha hydroxy acids, and dicarboxylic acids. Previous studies have shown that these compounds have antibacterial activity (32-35). In our study, we did not associate the observed antibacterial activity with the S-45 metabolite content. The obtained CFSs contain numerous primary and secondary metabolites. This difference in metabolite content suggests that the observed activities may not be attributed to a single compound alone. The combined effects of these synergistic or antagonistic metabolites may explain the observed activities (36, 37).

In a study conducted by Kachhadia et al., *B. cereus* RC1 extract was found to produce cyclic dipeptides such as cyclo (L-Leu-

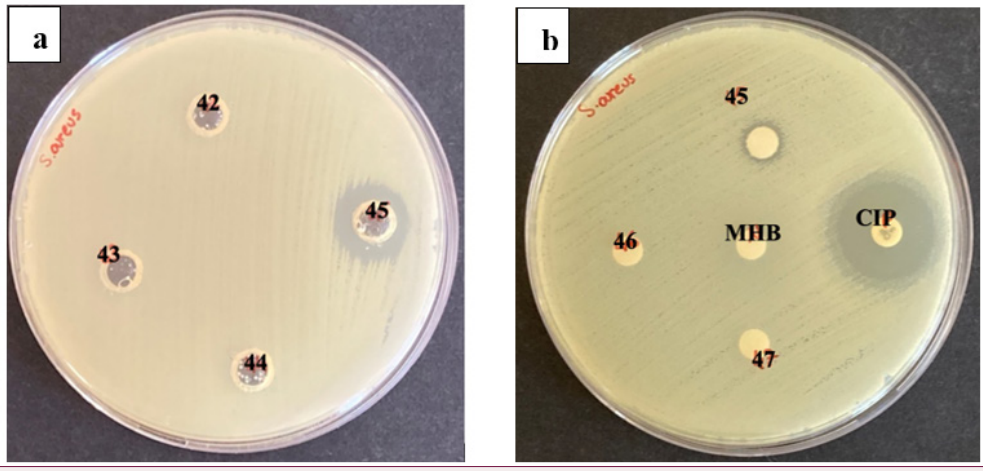

Figure 1. The test results of the S-45 using the (a) agar well diffusion method and (b) agar disk diffusion method (MHB: negative control, CIP: Ciprofloxacin-positive control).

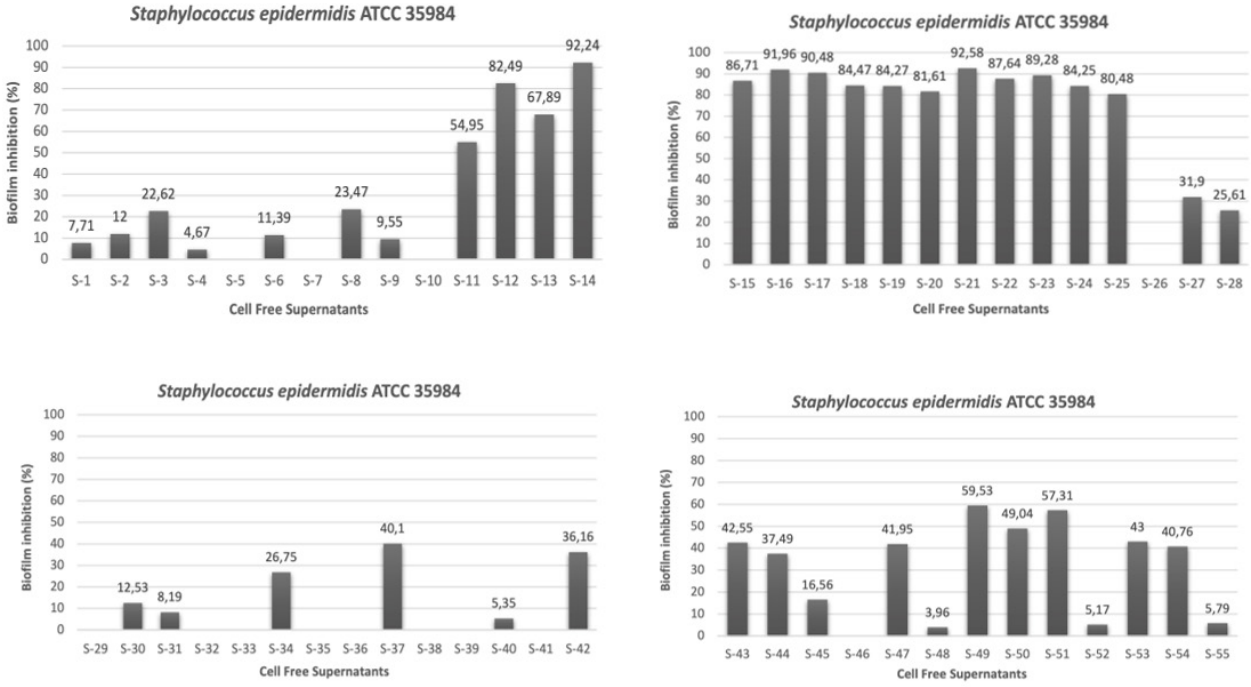

Figure 2. The biofilm inhibition percentages of the CFSs against *S. epidermidis* ATCC 35984.

L-Pro) and diketopiperazines, which are key metabolites responsible for anti-QS activity. These metabolites significantly inhibited quorum sensing and biofilm formation (38). In our study, only sample S-37 exhibited anti-QS activity. However, we could not associate the observed antibacterial activity with the metabolite content of S-37. The presence of similar metabolites in our samples further supports the potential of *Bacillus* sp. extracts to disrupt quorum sensing pathways and reduce bacterial virulence.

In another study, an antimicrobial peptide obtained from the *Bacillus* CBSMS07 strain was found to inhibit biofilm formation by reducing microbial cell density on the surface of *P. aeruginosa* and *E. coli* (39). Additionally, Moryl et al. reported that a filtrate from *B. subtilis* I'1a containing surfactin, iturin, and fengycin lipopeptides exhibited strong antibiofilm activity against urinary tract pathogens, possibly due to the synergistic effect of these compounds (40). In our study, we found that compounds belonging to amino acids and peptides, hydroxy acids and derivatives, and fatty acyls exhibited a high positive correlation with antibiofilm activity against *P. aeruginosa* PAO1

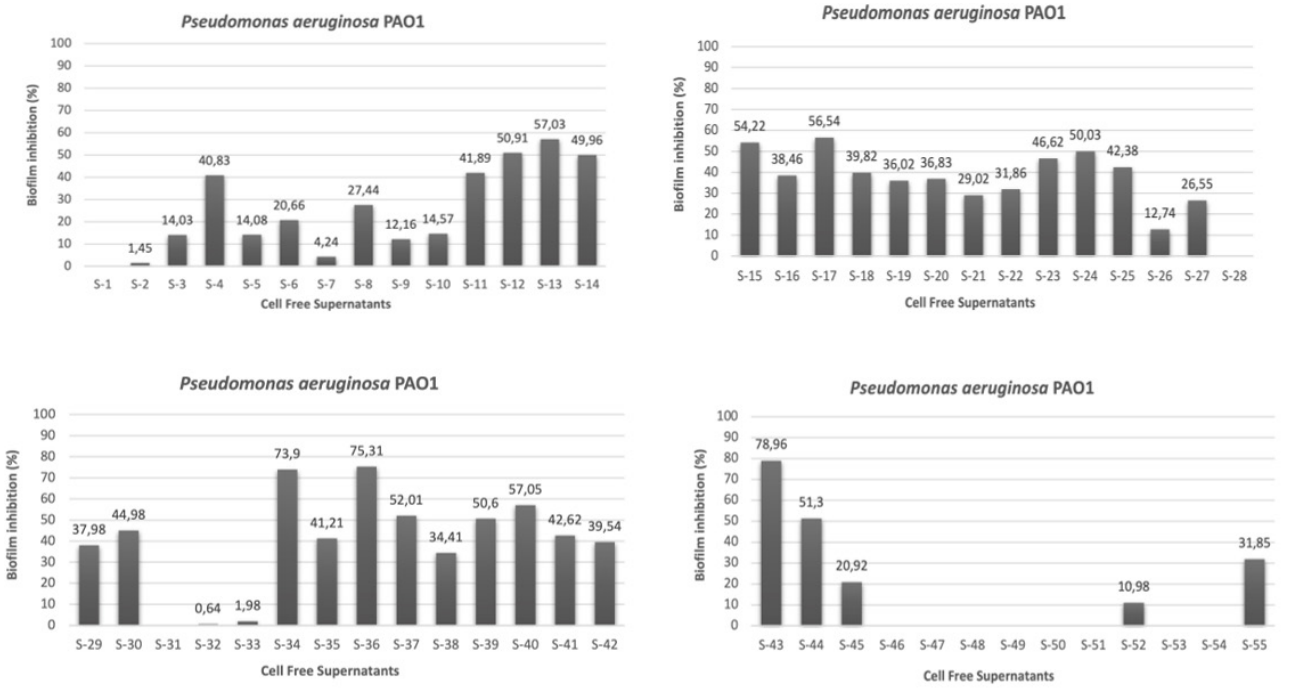

Figure 3. The biofilm inhibition percentages of the CFSs against *P. aeruginosa* PAO1.

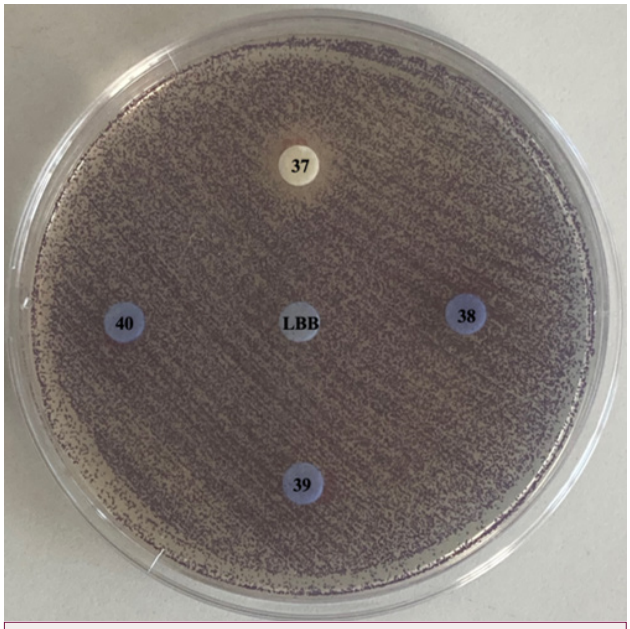

Figure 4. The anti-QS activity of S-37.

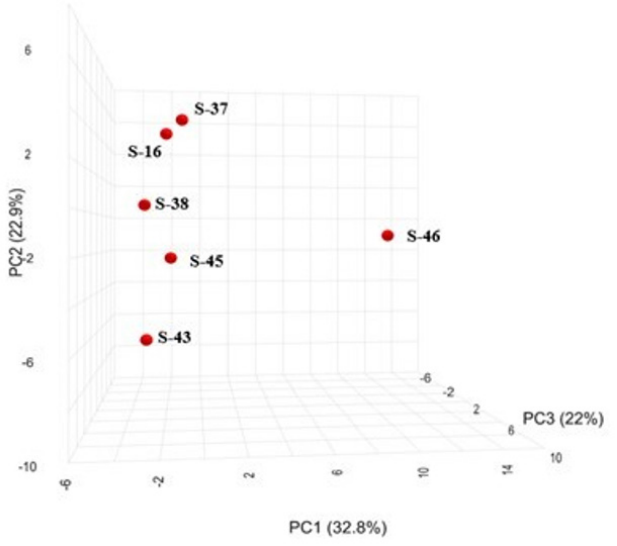

Figure 5. 3-D PCA graphs of S-16, S-37, S-38, S-43, S-45, S-46.

and *S. epidermidis* ATCC 35984. Malic acid, previously shown to have antibiofilm activity, also displayed a high positive correlation with antibiofilm activity in our study (41, 42). Additionally, amino acid-peptide group compounds with high positive correlation were identified in our correlation studies on *S. epidermidis* ATCC 35984, which is consistent with findings from other studies (43, 44).

In recent years, numerous peptides that exert strong inhibitory effects against microbial biofilms have been identified. This is significant because infections associated with biofilms are often not effectively eliminated by antibiotics alone (45). Cyclo (L-leucyl-L-propyl), a cyclic dipeptide, has been reported to dose-dependently inhibit the biofilm of *S. epidermidis* and *L. monocytogenes*, with an inhibition rate of more than 80% (46). Gowrishankar et al. showed that the same compound inhibited

the production of virulence factors such as exopolymer, biofilm, lipase, and protease at different levels against *S. marcescens* (46). Yu et al. showed that the active compound Cyclo (L-Tyr-L-Pro) reduced biofilm formation of *P. aeruginosa* PAO1 by 48% at 0.5 mg/mL and inhibited the QS system of *P. aeruginosa* (47).

Dostert et al. investigated the antibiofilm activity of amino acid-containing compounds and their combination with ciprofloxacin for synergistic activity. It was reported that amino acids can disperse mature biofilms and inhibit the biofilm formation of *S. aureus* (48). According to our findings, L-asparagine (r^2=0.95), N-acetyl-DL-serine (r^2=0.85), L-prolinamide (r^2=0.83), and N-acetyl-L-leucine (r^2=0.78) compounds in the amino acid group showed high correlation with *S. epidermidis ATCC 35984*. We also found that homocystine, a derivative of alpha amino acids, had a high positive correlation with *P. aeruginosa* PAO1 (r^2=0.93) and *S. epidermidis* ATCC 35984 (r^2 =0.74).

In the study, which demonstrated the presence of various fatty acyl compounds through GC-MS analysis and their effects on the QS system (LasI), it was found that the extract obtained from *Streptomyces griseoincarnatus* HK 12 showed antibiofilm activity against *P. aeruginosa* and *S. aureus* with approximately 80% inhibition at 100 µg/mL (49). According to our correlation results, lactitol, a fatty acid group compound, showed a more negative correlation with antibiofilm activity. However, palmitic acid, as determined by GC-MS analysis showed positive correlation (r^2=0.66) against *S. epidermidis* ATCC 35984 strain.

The similarities between the isolates were based on metabolite variations determined using PCA graphs. Subsequently, we performed correlation analyses between the metabolite profiles of chosen CFSs, selected based on their activities, and the percentage of biofilm inhibition results obtained against *P. aeruginosa* PAO1 and *S. epidermidis* ATCC 35984. As a result of this correlation analysis, we identified metabolites that exhibited a strong positive correlation. These results underscore the potential of specific metabolites in *Bacillus* sp. extracts for combating biofilm-related infections and highlight the need for further research to isolate and test individual compounds.

CONCLUSION

Antibacterials are essential therapeutic agents for treating bacterial infections. The rising incidence of antibiotic resistance has accelerated the search for new agents with different mechanisms of action that can be safely and effectively used in treatment. *Bacillus* species are known to produce various compounds with biological activities. In our study, we investigated the antibacterial, antibiofilm, and anti-QS activities of CFSs obtained from soil bacteria and identified metabolites potentially responsible for these activities. Our correlation studies revealed *Bacillus* sp. metabolites that were positively correlated with antibiofilm activity. The results of this study are expected to lead to the discovery of promising new therapeutics.

Ethics Committee Approval: Only cell culture material was used in this study and no living material that would require ethics committee approval was used.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study – G.B.Y., E.M.G., N.B.B., E.N., E.K., M.E.; Data Acquisition – G.B.Y., M.E., N.B.B; Data Analysis/Interpretation - G.B.Y., E.M.G., E.N., E.K., M.E.; Drafting Manuscript – G.B.Y., E.M.G., E.N., E.K., M.E.; Critical Revision of Manuscript – G.B.Y., E.M.G., N.B.B., E.N., E.K., M.E.; Final Approval and Accountability – G.B.Y., E.M.G., N.B.B., E.N., E.K., M.E.

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