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Research Article

Locally-isolated protease-producing *Bacillus* spp. from soil inhibits biofilm formation of *Staphylococcus aureus*

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ABSTRACT

Pathogens form biofilms to increase their resistance to environmental stress and antibacterial compounds. The rhizosphere is a rich source of microorganisms producing industrially important compounds including those with antimicrobial and biofilm inhibitory activities. Four isolates from soil collected from Taguig City, Philippines, were subjected to phenotypic and genotypic characterization, screening for protease production, and biofilm inhibition assays. Colony morphology and microscopic analyses indicated the isolates were putative *Bacillus* species. Upon DNA extraction, 16S rRNA gene was amplified, and based on their sequences, the isolates were confirmed to be *Bacillus* spp. Isolate AHP was *B. cereus*, isolate DJA was *Priestia megaterium*, formerly known as *B. megaterium* and isolates SJS and SKA were *Bacillus* spp.—all of which produced protease. Although the cell-free supernatants (CFS) of the isolates did not inhibit the growth of *Staphylococcus aureus* 1258, *Citrobacter freundii* ATCC24864, *Salmonella* Typhimurium ATCC13311, *Escherichia coli* ATCC11229, and *E. coli* O157:H7, biofilm formation of *S. aureus* was inhibited by all CFS, with *B. cereus* AHP showing the highest biofilm inhibition at 46%, followed by *Bacillus* sp. SKA (39%), *P. megaterium* DJA (36%), and *Bacillus* sp. SJS (31%). Even though further studies are warranted, the bioactivities of these isolates indicate potential use for pharmaceutical purposes due to their ability to produce protease and inhibition of biofilm formation of a common bacterial pathogen.

Keywords:
Bacillus spp., Biofilm inhibition, Enzyme screening, Protease, *Staphylococcus aureus*

1. INTRODUCTION

Antimicrobial resistance cases among bacteria pose a significant threat globally to health care systems, food security, and agricultural and veterinary fields. Repetitive studies focusing on synthetic antibiotics targeting bacterial gene expression, cell wall synthesis, and nucleic acid synthesis impede the progress in developing new antibiotics[1]. Exacerbating the problem of resistance is the ability of bacterial cells to employ a coordinated lifestyle wherein they communicate and establish a community organized in extrapolymeric substances (EPS) known as biofilms[2]. This complex lifestyle provides additional resistance to antibacterial compounds through the barrier conferred by EPS, as well as changes in the physiology of the cells which render compounds inactive against their targets[3]. Bacterial proteases may offer a unique approach to antimicrobial resistance. Intracellular proteases are crucial in cellular protein pool, protein turnover, and differentiation, whereas extracellular proteases can break down protein through hydrolysis[4]. Notable proteolytic bacteria such as *Staphylococcus*, *Pseudomonas*, *Clostridium*, and *Bacillus* can hydrolyze proteins through their secretion of proteinases—an enzyme capable of interfering with biofilm formation, one of the bacterial virulence factors[5].

Bacillus spp. are rod-shaped Gram-positive bacteria present in the soil, some of which have been found to be involved in fermentation, although their application has expanded to other industries. Currently, some strains have

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been engineered to manufacture vitamin B2, poly-γ-glutamic acid (γPGA), nucleotides, and other secondary metabolites, including enzymes applied in various fields such as cosmetics, agricultural, and pharmaceutical industries, hence their potential as microbial cell factories[6]. Examples include *B. halodurans* and *B. invictae* that produce alkaline proteases for stain removal and deproteinization for chitin extraction, respectively[7-8], *B. amyloliquefaciens* that produce serine fibrinolytic proteinase used as a thrombolytic therapy alternative[9], and *B. licheniformis* producing amylase used in food and beverage industries[10]. Chemical engineering, microbiology, and molecular biology are integrating strategies to further explore and take advantage of their capabilities in producing safe, revolutionary, and multifunctional products.

Rhizosphere is the immediate narrow soil region around the plant root area. It is physically and chemically influenced by the plant root and its exudates such as carbohydrates, phenolic compounds, phytohormones, and organic acids that induce favorable conditions for microbial growth and survival, hence increasing microbial activity in the said region[11]. Rhizosphere is a rich source of microorganisms, especially bacteria that can exhibit a wide range of biological antibacterial and antifungal activities and produce compounds with biofilm inhibitory activities[12], attributed to their use of rhizodeposits from plants such as amino acids and polymeric carbohydrates as energy and carbon sources for their metabolic activities[11]. Considering these bioactivities of rhizospheric bacteria in the context of plant defense against pathogens, they may also be targeted for the discovery of drugs useful for pharmaceutical purposes.

Therefore, this study aimed to determine the antibacterial potential and biofilm inhibitory activity of proteolytic *Bacillus* spp. isolated from a rhizosphere soil sample of *Terminalia catappa* L. tree, which has important pharmacological and phytochemical properties[13], against common bacterial pathogens—*Staphylococcus aureus, Citrobacter freundii, Salmonella* Typhimurium, and *Escherichia coli.*

2. MATERIALS AND METHODS

2.1. Isolation and identification of *Bacillus* spp. from soil

Twenty-five grams (25 g) of soil was collected approximately five centimeters (5 cm) deep in the rhizosphere of a *Terminalia catappa* L. tree in Bicutan, Taguig City, Philippines. The soil sample collected was stored in a sterile resealable plastic bag. Immediately upon arrival in the laboratory, the sample was processed by homogenizing with 0.1% peptone water. Serial dilution of up to 10^{-4} was performed, and each dilution was spread on Actinomycete Isolation Agar (AIA) (since this study initially aimed to isolate actinomycetes). Plates were incubated for 24 h at 35°C. Isolates based on their colony morphologies were selected and isolated by streaking on tryptone soya agar (TSA) plates. Once pure cultures were obtained, they were stored at 4°C until further use.

Identification of the isolates was performed through phenotypic and genotypic characterization. Colony and cellular morphologies were noted through visual observation and microscopy, respectively. For the genotypic identification, genomic DNA of the isolates were extracted using Vivantis GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia) based on the manufacturer's protocol. Thereafter, PCR amplification of 16S rRNA gene was performed using MyTaq Red HS PCR Mix (Bioline, USA) using the primers 27F (5'-AGAGTTT GATCMTGGCTCAG-3') and 1492R (5'-TACGGYTA CCTTGTTACGACTT-3'). PCR products were run on 1% agarose gel electrophoresis for verification. Thereafter, amplicons were sent to Macrogen, Korea for capillary sequencing. The sequences were analyzed using NCBI BLASTn and phylogenetic trees were constructed using the Neighbor-Joining Method using MEGA X phylogenetic software. Sequences were also deposited to the NCBI Genbank database.

2.2. Qualitative screening for protease production

Bacillus spp. isolates were streaked on TSA supplemented with 1% skim milk (SMA), and were then incubated for 24 h at 35°C. A clearing zone indicated protease activity[14].

2.3. Extraction of cell-free supernatants (CFS) of *Bacillus* spp. Isolates

Each *Bacillus* spp. isolate was grown in tryptone soya broth (TSB) tubes for 24 h at 35°C with shaking (150 rpm). Thereafter, the tubes were subjected to centrifugation at 10,000 rpm for 5 min at 4°C. The supernatants were collected then passed through a 0.2 µm syringe filter to obtain cell-free supernatants (CFS). These CFS were used for the succeeding assays.

2.4. Antibacterial assays

2.4.1. Qualitative colorimetric growth assay

The resazurin-based CellTiter-Blue® Cell Viability Assay (Promega, USA) was used to determine the effects of the CFS on the growth of the test organisms. Initially, cultures of the test organisms (local clinical isolate *Staphylococcus aureus* 1258, *Citrobacter freundii* ATCC24864, *Salmonella* Typhimurium ATCC13311, *Escherichia coli* ATCC11229, and *E. coli* O157:H7) were grown overnight in TSB at 35°C. The overnight cultures were then diluted to OD_{600nm} 0.05 for use as inocula.

Subsequently, in a 96-well microtiter plate, 160 µL of the inocula were placed into each well. Afterwards, 20 µL of the *Bacillus* spp. CFS were added. Finally, 20 µL of CellTiter-Blue® reagent were added as an indicator of cell viability. The plates were then incubated at 35°C for 18 h with shaking (150 rpm). A change in color from blue to pink was an indication of growth, while the absence of color change signified growth inhibition[15]. The assay was performed with four replicates.

2.4.2. Disk diffusion assay

Disk diffusion assay was performed to quantify the growth inhibitory activities of *Bacillus* spp. CFS against the test pathogens. First, overnight cultures of the test pathogens were diluted to OD_{600nm} 0.05 to prepare the inocula. Thereafter, a sterile cotton swab was moistened with the inocula to create a bacterial lawn onto the surface of TSA plates. Sterile blank disks were then impregnated with 20 µL *Bacillus* spp. CFS and then placed on the surface of TSA plates. After incubation at 35°C for 24 h, zones of inhibition, as indicated by clear halos around the disks, were measured.

2.4.3. Quantitative spectrophotometric growth assay

Overnight cultures of each test pathogen in TSB were first adjusted to OD_{600nm} 0.05. Thereafter, 180 µL of each culture were placed in the wells of a 96-well microtiter plate. Subsequently, 20 µL of *Bacillus* spp. CFS were added. The microtiter plates were then incubated at 35°C for 24 h with shaking (150 rpm). The absorbance values at OD_{600nm} for each well were measured using a microplate reader (Biotek 800TS)[16]. The assay was performed with four replicates.

2.5. Biofilm inhibition assay

Quantification of biofilm formation was performed according to the method described by Yatip et al. (2018) with few modifications[16]. Test pathogens which were grown in TSB overnight then adjusted to OD_{600nm} 0.05 served as the inocula for the assay. Initially, 180 µL of each inoculum were placed in the wells of a 96-well flat-bottomed microtiter plate. Subsequently, 20 µL of *Bacillus* spp. CFS were added. The plates were then incubated at 35°C for 24 h without shaking. Thereafter, the contents of the wells were carefully discarded, then washed with distilled water (DW) to remove planktonic cells. After drying for three hours at room temperature (RT), the wells were stained using 0.3% crystal violet for 15 min at RT. The wells were then washed with DW to remove the excess stain then dried for 2 h at RT. Stained biofilms in each well were dissolved with 200 µL 33% acetic acid for 15 min at RT. The absorbance values of each well at OD_{600nm} were measured using a microplate reader (Biotek 800TS). The assay was performed with four replicates.

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 2.0 software. To determine if there is a significant difference among the inhibitory activities of *Bacillus* spp. CFS, as well as compare them with the controls, one-way analysis of variance (ANOVA) was used. Tukey's post hoc test was also utilized to identify where the significant difference lied. Values were presented as means±standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Protease-producing *Bacillus* spp. were isolated from the rhizosphere of *Terminalia catappa* L.

Soil microbial communities, including actinomycetes and *Bacillus* spp., form beneficial interactions with plants. In addition to stimulating plant growth, a previous study found that the presence of soil microbial communities provided defense against pathogens, slowed the progression of disease, and lowered the intensity of disease symptoms[17]. Considering the relevance of plant-microbe interactions and since the goal of this work was to search for bacterial strains capable of producing compounds with proteolytic and antibacterial activities, this study targeted bacterial isolates from the rhizosphere of a native tree, *T. catappa* L., a plant reported to have bioactive properties[13]. Though initially aimed at isolating actinomycetes, hence the use of a selective media for the bacterial group, no colonies typical of actinomycetes grew on the media. Thus, the team decided to isolate colonies typical of *Bacillus* spp. since they are also commonly reported to have a wide range of bioactivities, notably antimicrobial and biofilm inhibitory activities, and can also be found in the rhizosphere[12]. A total of four isolates were selected due to their morphological characteristics typical of *Bacillus* spp. (Figure 1).

Furthermore, plant-microbe interactions occur through specialized components of root exudates, which plants produce to recruit favorable rhizosphere bacteria. Root exudates act as signals that affect interactions in rhizospheres by establishing symbiotic partnerships that aid plants in surviving stress, promoting growth, or triggering defenses against pathogens. Due to nutrient competition and the mechanisms that stimulate increased microbial activity in the rhizosphere, exudates are important determinants of microbial composition in the rhizosphere[17].

In the rhizosphere of the majority of plants, *Bacillus* spp. are the most dominant bacteria where they play a fundamental ecological role in sustaining the soil ecosystem, recycling soil nutrients, improving soil conditions that are beneficial for crop production, and providing

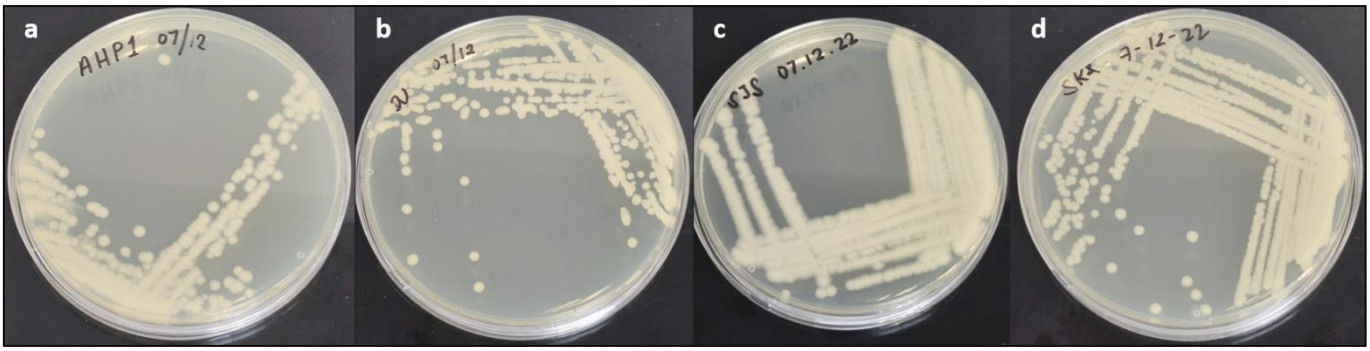

Figure 1. Isolates (a) AHP, (b) DJA, (c) SJS, and (d) SKA on tryptone soya agar.

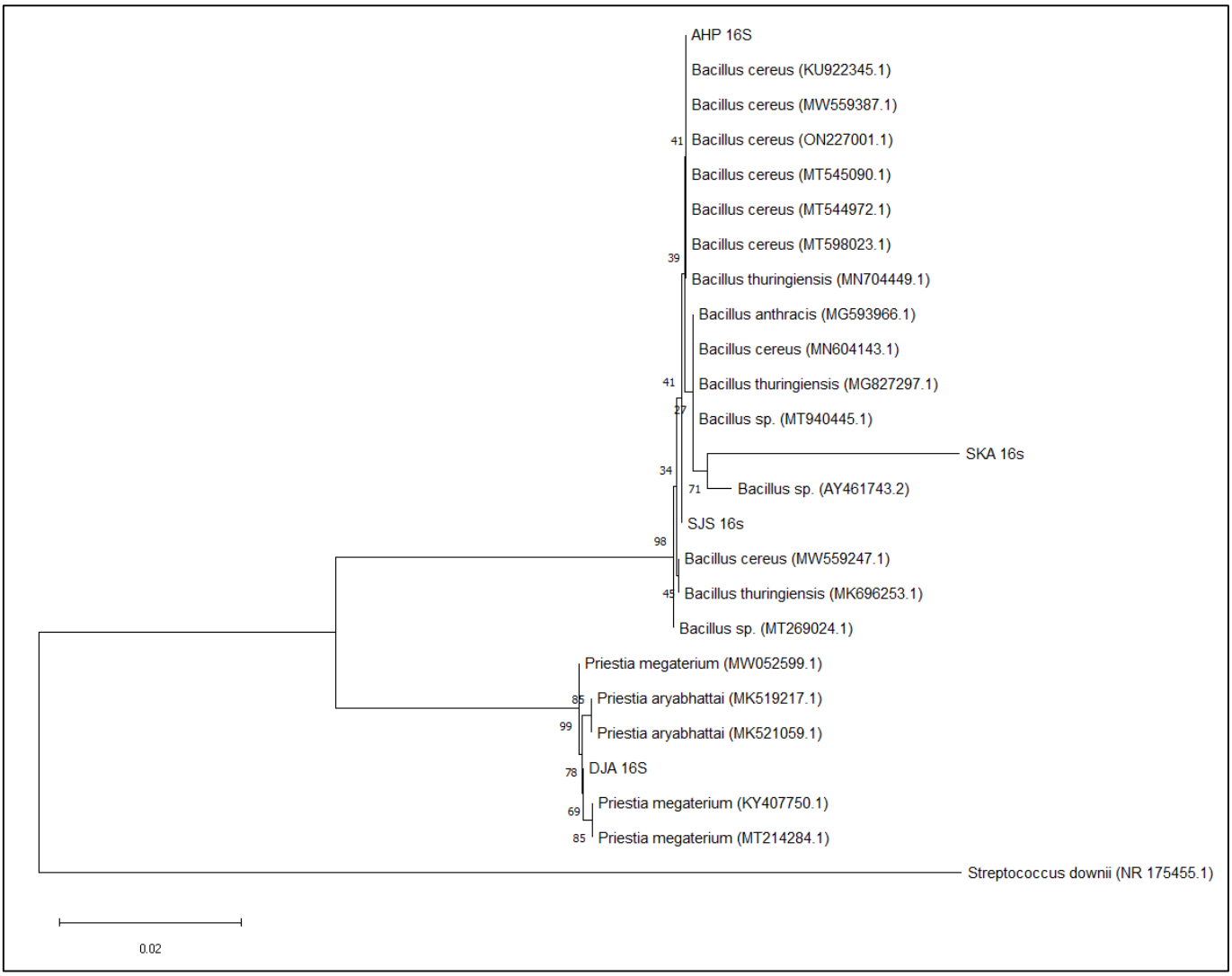

Figure 2. Phylogenetic analysis of 16S rRNA genes of isolates AHP, DJA, SJS, and SKA constructed with the Neighbor-Joining Method using the MEGA X phylogenetic software.

Table 1. NCBI accession numbers of the 16s rRNA gene of the isolates.

Isolate	Length	NCBI Accession Number
Bacillus cereus AHP	1403 bp	OP295072.1
Priestia megaterium DJA	1416 bp	OP290294.1
Bacillus sp. SJS	1391 bp	OP341432.1
Bacillus sp. SKA	1014 bp	OP341740.1

protection from pathogens and other stressors[18]. This makes the rhizosphere an ideal location for collecting *Bacillus* spp. with bioactivities that are medicinally valuable. Hence, this study used the ability of *Bacillus* spp. to confer additional resistance against pathogens in plants as a basis for targeting them in the discovery of bioactive substances with pharmaceutical importance. The isolates were further identified genotypically through the amplification and sequencing of their 16S rRNA gene. Phylogenetic analyses identified AHP as *B. cereus*, DJA as *Priestia megaterium*, formerly known as *B. megaterium*, and isolates SJS and SKA as *Bacillus* sp. (Figure 2). Gene sequences were deposited in the NCBI Genbank and can be accessed via their respective accession numbers listed on Table 1.

This dominance is due to the antagonistic activities of *Bacillus* spp. In an experimental study previously reported, most *Bacillus* spp. exhibited antagonistic activities toward *B. mycoides*—this activity was attributed to the synthesis of bacteriocins[19]. Bacteriocins are proteins synthesized by bacteria that restrict the growth of other bacteria that are closely related to them and may be effective in the fight against pathogenic and antibiotic-resistant bacteria. *Bacillus* spp. has extensive antimicrobial properties due to the production of bacteriocins[20]. In addition to their antagonistic activities, the ability of *Bacillus* spp. to generate robust, resistant endospores make them highly tolerant to environmental stressors and exceptionally adaptable which then contributes to their dominance in the rhizosphere[21].

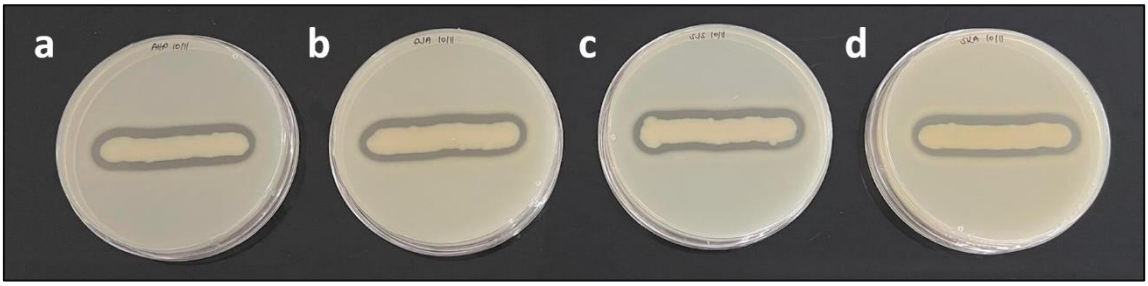

Figure 3. Protease production of (a) *Bacillus cereus* AHP, (b) *Priestia megaterium* DJA, (c) *Bacillus* sp. SJS, and (d) *Bacillus* sp. SKA on skim milk agar.

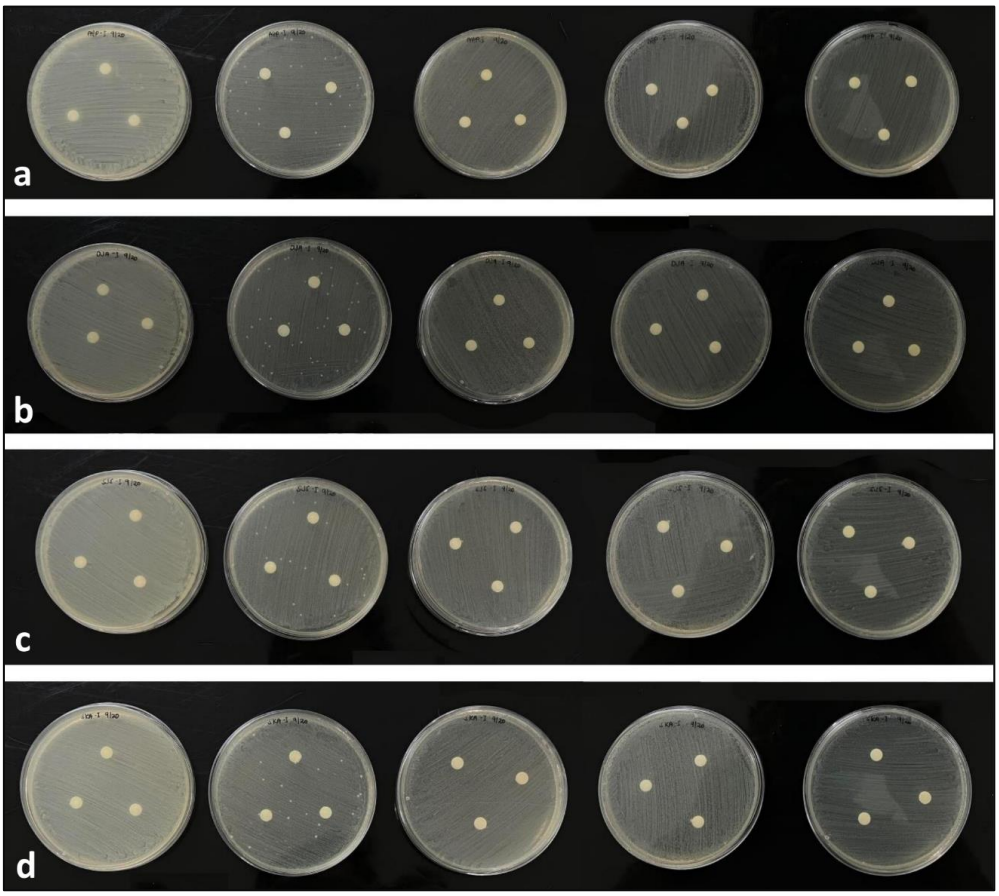

Figure 4. Disk diffusion assay using cell-free supernatants of (a) *Bacillus cereus* AHP, (b) *Priestia megaterium* DJA, (c) *Bacillus* sp. SJS, and (d) *Bacillus* sp. SKA against (from left to right) *Staphylococcus aureus* 1258, *Citrobacter freundii* ATCC24864, *Salmonella* Typhimurium ATCC13311, *Escherichia coli* ATCC11229, and *Escherichia coli* O157:H7.

Microorganisms also produce proteases—an enzyme that catalyzes protein hydrolysis. Although this enzyme is ubiquitous across all organisms, microbial proteases have greater economic value due to its convenience, safety, and stability compared to those of plants and animals. Furthermore, its wide applications in the industrial sector such as in the food, pharmaceutical, textile, and cleaning industries, etc., adds up to its commercial value. Despite its importance, there are only a few commercial protease producers, one being *Bacillus* spp. Several literatures already discussed the ability of *Bacillus* spp. to produce proteases. For instance, the study conducted by Patil and Jadhav (2017) showed that 28 *Bacillus* spp. isolates, especially the *B. cereus, B. licheniformis,* and *B. megaterium*, demonstrated excellent protease enzyme production after 24 and 72 h[22]. Another study performed by Pant et al. (2015) analyzed which factors and conditions affect the protease activity of *B. subtilis*. The results emphasized the influence of the composition of the medium the isolate was grown, the temperature, pH, inoculum load, as well as the length of incubation towards the production of protease[23].

Since proteases can tolerate extreme conditions, and that soil microbial proteases have the capability to inhibit plant pathogens and parasites, they are seen as good biocontrol agents[24]. To assess protease production, various methods were used and one of them is growing cultures on SMA. The clearing zones on SMA indicate the ability of *Bacillus* spp. to produce protease (Figure 3).

3.2. *Bacillus* spp. isolates from soil inhibit biofilm formation of *Staphylococcus aureus* without inhibiting its growth

Biofilm formation increases the resistance of bacterial pathogens against environmental stress and antibacterial compounds[2], hence, this study focused on the search for bacterial isolates from soil capable of interfering with biofilm formation. To ensure that the observed changes in the biofilm biomass were due to the inhibition of biofilm formation and not because of the bactericidal properties of the CFS, growth inhibition assays were carried out. Figure 4 shows that based on the disk diffusion assay, all *Bacillus* spp. CFS were non-growth inhibitory. Furthermore, based on CellTiter-Blue® assay, all isolates did not inhibit the growth of the test pathogens, as indicated by change in color of the wells from blue to pink[15].

Prior to the quantification of biofilm formation, planktonic cell growth of all test pathogens upon treatment with *Bacillus* spp. CFS was also measured spectrophotometrically at OD_{600nm}. The absorbance values of the control and the wells treated with *Bacillus* spp. CFS were not significantly different—indicating no growth inhibition. Despite this, there was an observed decrease in biofilm formation in one of the test pathogens. This is quite similar to the results of the study by Qiu et al. (2022) wherein *B. subtilis* H28 did not inhibit the growth of *S. aureus* but inhibited its biofilm formation[25]. Interestingly, results of the quantification of biofilm biomass showed that the formation of biofilms by Gram-negative strains used in this study were not affected by all the CFS. However, there were significant decreases in the biofilm biomass formed by *S. aureus* 1258 upon treatment with the CFS (Figure 5). Specifically, *B. cereus* AHP yielded the greatest biofilm inhibition with 46%, followed by *Bacillus* sp. SKA with 39%, *P. megaterium* DJA with 36%, and *Bacillus* sp. SJS with 31%.

There are already previous studies reporting that the CFS from *Bacillus* spp. inhibits biofilm formation of *S. aureus*. In a study by Raffaelli et al. (2022), CFS from *B. cereus* ILBB55 almost completely eradicated biofilm formation in *S. aureus*. They have concluded that the biofilm inhibitory activity of the tested *B. cereus* strain may be due to the wide plethora of antimicrobial peptides present in the CFS based on the investigation of the biosynthetic gene clusters found in the genome of the aforementioned strain[26]. Furthermore, combining antibiotics with *Bacillus* spp. CFS, which were shown to inhibit biofilm formation, rendered resistant strains of *S. aureus* susceptible to the combined treatment-emphasizing the importance of antibiofilm compounds in controlling bacterial pathogenesis[27].

A similar study in 2021 by Zhang et al. reported an increase in the susceptibility of *S. aureus* against antibiotics upon treatment with *B. subtilis* CFS. This observation was attributed to the capability of the CFS to inhibit biofilm formation, particularly through the suppression of genes involved in quorum sensing (QS), biofilm formation, and adhesion. Ultimately, they found out that *S. aureus* burden *in vivo* was significantly reduced[28]. Another study using *B. subtilis* H28 CFS showed that biofilm formation by *S. aureus* was significantly reduced due to the modulation of its QS system[25].

A separate study that evaluated *S. aureus* biofilm formation *in vitro* reported that DNA of biofilm samples from medical device substrates treated with *B. velezensis* AP183 prior to subsequent *S. aureus* colonization revealed a significant relative abundance of *B. velezensis*, at 96.5% against *S. aureus*, at 3.5% based on ribotype relative abundance data[29]. The same study showed that *B. velezensis* AP183 could disrupt formed biofilms of *S. aureus* and noted the application of beneficial bacterial biofilms, which could hinder the adhesion and biofilm formation of pathogenic bacteria such as *S. aureus* as a prophylactic advantage.

Though the *Bacillus* spp. isolates used in the current study were found to inhibit biofilm formation of Gram-positive *S. aureus* without inhibiting biofilm formation of the Gram-negative test pathogens *C. freundii, Salmonella* Typhimurium, and *E. coli*, it is not deduced from here that *Bacillus* spp. favor Gram-positive biofilm inhibition over Gram-negative. Tazehabadi et al. (2021)

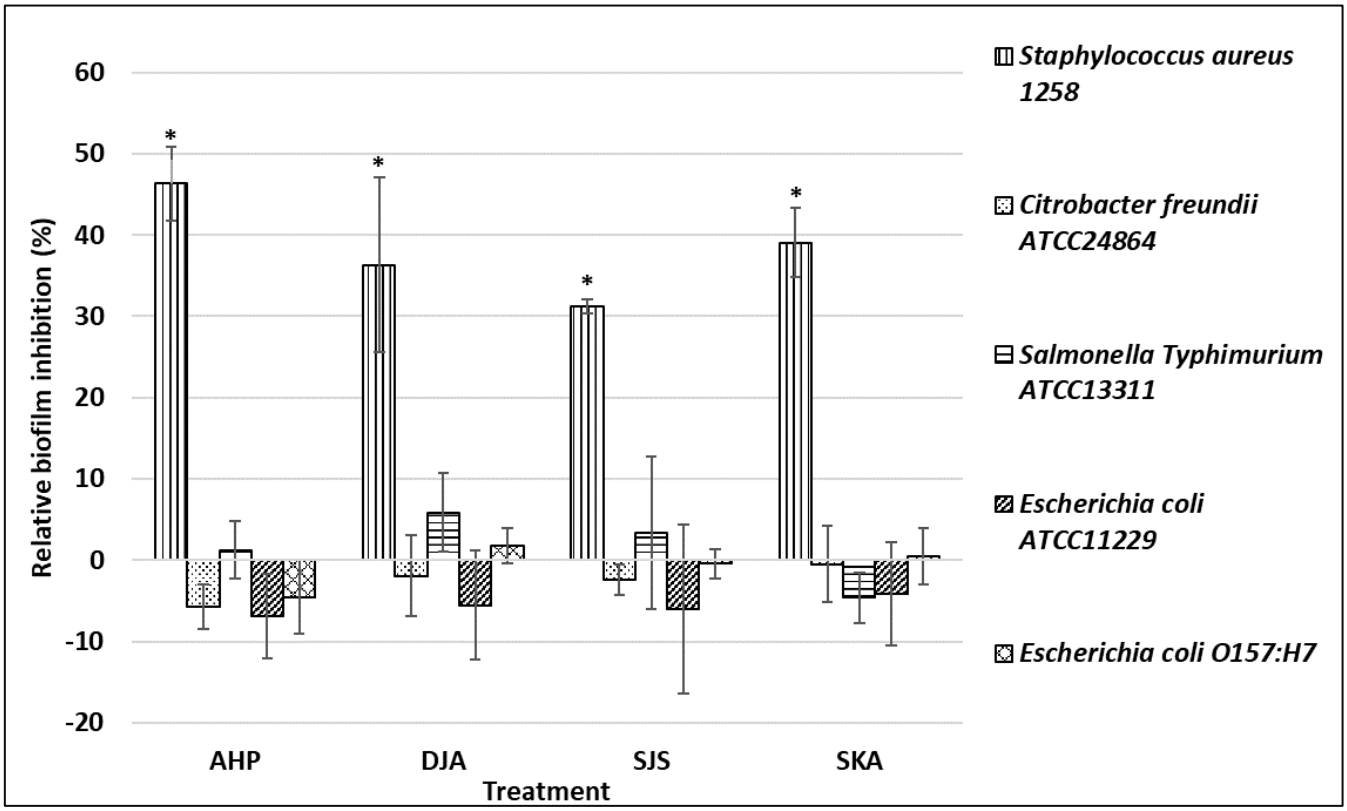

Figure 5. Cell-free supernatants from *Bacillus cereus* AHP, *Priestia megaterium* DJA, *Bacillus* sp. SJS, and *Bacillus* sp. SKA significantly inhibited biofilm formation of *Staphylococcus aureus* 1258 relative to the control. Values are presented as means±standard deviation. *denotes significant difference against the control.

reported *B. subtilis* KATMIRA 1933 and *Bacillus amyloliquefaciens* B-1895 CFS as having biofilm-inhibitory activities against *Salmonella* spp. without inhibiting their growth in co-cultures, indicating the mechanism of action of the *Bacillus* strains used as affecting QS, not being bactericidal, and that this activity may be due to the presence of peptides and bioactive compounds such as subtilosin produced by the two *Bacillus* spp[30]. Separately, Mahdhi et al. (2018) reported that exopolysaccharides produced by *Lactobacillus plantarum* and *Bacillus* spp. were able to reduce *E. coli* biofilm formation[31]. These studies note the importance of *Bacillus* spp. and their products as having probiotics and antibiotic applications in controlling Gram-negative bacterial pathogens. While there are studies reporting that *Bacillus* spp. inhibit biofilm formation in Gram-negative bacteria[30-32], it is noteworthy that several studies screening for their inhibitory activities against biofilm formation yielded positive results against Gram-positive bacteria, particularly *S. aureus*.

Algburi et al. (2016) stated that the antimicrobial substance, subtilosin, from *B. subtilis* KATMIRA1933 was able to affect QS by reducing the level of production of autoinducer-2. The same strain was later found to have antibiofilm activity against resistant strains of *S. aureus*[33]. Another study that reported the biofilm-inhibitory activity of *B. subtilis* CFS through its suppression of QS, biofilm formation, and adhesion genes noted the need for further investigations to characterize the exact compounds produced by the CFS that has this activity[28].

Quorum sensing in Gram-positive bacteria is majorly due to the accessory gene regulator (Agr) system, and such is found to be ubiquitous in staphylococci. This protein-mediated quorum-sensing system is important in regulating the expression of virulence factors (adhesins, toxins, and degradative exoenzymes) and the formation of biofilms in *S. aureus* where the former provides this opportunistic. Gram-positive bacteria with the timely expression of factors for the development of infections into acute diseases while the latter is more associated with chronic infections[34]. Marti et al. (2010) found that proteases Aur and SspA overexpression in *S. aureus* protein-mediated biofilm formation led to the degradation of Bap (Biofilm-associated proteins), inhibiting the formation of this matrix[35]. Interestingly, targeting the Agr QS system and other QS regulators has also led to reduced biofilms. This was confirmed by Chen et al. (2016), who reported the molecular mechanism of antibiofilm activity of baicalein against *S. aureus* as relating to the downregulation of *AgrA*, *RNAIII*, and *sarA* QS system regulators[36].

Although further investigations are needed to identify and characterize the proteases produced by the *Bacillus* spp. isolates used in the current study and their mechanism of action molecularly, it is known that *Bacillus* spp. proteases play a role in biofilm inhibition. Specifically, some of these extracellular proteases exhibit

high proteolytic activity against extracellular polymeric substances (EPS), affecting the physical integrity and slime layers of biofilms. It should also be noted that proteases, being enzymes, are substrate-specific proteins so despite the high-yield concentration produced by the *Bacillus* spp., some components such as lipids, other extracellular DNA, and various proteins present on the biofilm will not be degraded resulting in low or varied antibiofilm activity against different microorganisms[37]. Neutrase and alcalase, isolated from *B. amyloliquefaciens*, and *B. licheniformis*, respectively, have been found to have antibiofilm activities against *S. aureus* and *S. epidermidis*[38]. Both are endoproteases where the former was found to have varied biofilm-inhibitory reactivity between the two *Staphylococcus* spp., and such difference was attributed to the variation of the expressed proteins of the *Staphylococci* biofilms including Bap (Biofilm-associated protein), Aap (accumulation-associated proteins), Sas (*S. aureus* surface protein) or Ses (*S. epidermidis* surface protein). Thus, proteases produced by the isolates in this study may have contributed to the observed biofilm inhibitory activities. However, because the relationship between the *Bacillus* spp. protease production and its antibiofilm activity depends on whether the enzymes have biofilm components that will serve as substrates, further investigation on the characterization of the proteases produced and the evaluation of the target microorganism's biofilm components are necessary to prove such hypothesis.

Biofilm formation, established by cell-to-cell communication, impedes the penetration of antibiotics, and makes cells more robust against antimicrobial compounds, ultimately making pathogens more resistant to antibiotics[2], which is why there is ongoing research on novel antimicrobial compounds which target the formation of pathogenic biofilms. This strategy of targeting biofilm formation and QS systems is also considered advantageous compared to the typical antimicrobial treatment against bacterial growth since the latter brings about selective pressure, leading to the development of resistant strains[39].

Thus, searching for compounds capable of interfering with biofilm formation-a mechanism vital to bacterial pathogenesis may significantly contribute to combating bacterial infections; and the results of this study suggest that *Bacillus* spp. isolated from the rhizosphere may serve as a good source of these compounds.

4. CONCLUSION

In this study, four *Bacillus* spp. isolates, identified through phenotypic characterization and phylogenetic analysis, were obtained from a rhizospheric soil sample. Based on the results of the assays, the CFS of the four proteolytic *Bacillus* spp. isolates were able to disrupt the formation of *S. aureus* biofilm without affecting its growth. Overall, these indicate the potential of locally-isolated *Bacillus* spp. from soil as sources of bioactive compounds with pharmaceutical importance. Future studies focusing on testing these isolates against other clinically-important microorganisms, the identification and characterization of the bioactive compounds they produce, and the evaluation of their mechanisms of action may contribute in the development of new compounds which may control the pathogenicity and virulence of *S. aureus*.

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Conflict of Interest
None to declare.

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DJDA - Data Curation, Investigation, Resources, Visualization, Writing-original draft, Writing-review and editing
SKA - Data Curation, Investigation, Resources, Visualization, Writing-original draft, Writing-review and editing
NLN - Data Curation, Investigation, Resources, Visualization, Writing-original draft, Writing-review and editing
AHP - Data Curation, Investigation, Resources, Visualization, Writing-original draft, Writing-review and editing
JPGJ - Funding Acquisition, Resources, Software
REDC - Formal Analysis, Investigation, Validation, Writing-review and editing
JPMG - Conceptualization, Data Curation, Formal Analysis, Methodology, Supervision, Validation, Writing-original draft, Writing-review and editing

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