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Supernatant of plant-associated bacteria potency against biofilms formed by foodborne pathogen and food spoilage bacteria

Gabriella Gandasurya[1], Diana Elizabeth Waturangi[2*], Nurmaya Papuangan[3], Nurhasanah[3] and Pande Gde Sasmita Julyantoro[4]

Abstract

Objectives Food products are often contaminated by pathogens and spoilage bacteria. Most of them can form biofilms, a community of cells embedded in protective extracellular matrix layers resistant to harsh conditions, including antibiotics. Therefore, alternative antibiofilm agents are required to overcome biofilm formation. This study aims to determine and quantify the antibiofilm activity of supernatants from plant-associated bacteria against biofilms of foodborne pathogen and food spoilage bacterium, namely *Bacillus cereus* and *Bacillus subtilis*.

Results Plant-associated bacteria (PAB) have shown promising antibiofilm activities against biofilm-forming pathogens in previous studies. Thirteen PAB isolated from Ternate, Indonesia were used in this study. Supernatants of PAB were subjected to antimicrobial activity and quorum quenching detection, both using the well diffusion method. Four supernatants inhibited the growth of *B. subtilis*, but none affected the growth of *B. cereus*. Eight supernatants were able to disrupt the quorum sensing system of an indicator bacterium, wild-type *Chromobacterium violaceum*. Biofilm inhibition and destruction were quantified using 96-well microplates. The highest biofilm inhibition and destruction activities of PAB supernatants against each of *B. cereus* and *B. subtilis* biofilms were > 76%, and were later confirmed by light microscope and scanning electron microscope. Brine shrimp lethality assay (BSLA) was conducted and revealed that the selected PAB supernatants were non-toxic. The 16S rRNA gene of PAB were sequenced and they showed similarities to *Bacillus*, *Priestia*, and *Chryseobacterium*. Compounds in the supernatants were determined by GC–MS which revealed contents of fatty acids, ethyl esters, and diketopiperazines. Therefore, PAB supernatants have potential as antibiofilm agents against biofilm formed by *Bacillus cereus* and *Bacillus subtilis*.

Keywords Biofilm, Antibiofilm, Plant-associated bacteria, Foodborne pathogen, Food spoilage bacteria

*Correspondence:
Diana Elizabeth Waturangi
diana.waturangi@atmajaya.ac.id
[1] Department of Food Technology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jalan Raya Cisauk Lapan, Tangerang, Banten 15345, Indonesia
[2] Department of Master in Biotechnology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia
[3] Department of Biology Education, Faculty of Teacher Training and Education, Khairun University, Ternate 97728, Indonesia
[4] Department of Aquatic Resources Management, Faculty of Marine Science and Fisheries, University of Udayana, Denpasar, Bali 80361, Indonesia

Introduction

Food safety is a major concern in food industries due to the global widespread of unsafe food which poses threats to human health. Issues regarding food safety may be the results of chemical or microbiological hazards and other challenges, such as poor hygiene of food handlers, unsanitary food preparation, raw or undercooked food, improper food storage, etc. This leads to bacterial contamination, for example *Bacillus cereus* and *Bacillus subtilis* [1]. They can survive high food processing temperatures due to their endospores. *B. cereus* is a foodborne pathogen that causes mild or severe food poisoning via toxins [2]. It is found in milk, infant foods, and is a potential risk in the food industry [3, 4]. World Health Organization (WHO) reported an estimated 600 million foodborne illnesses, mostly diarrheal cases, and 420,000 deaths in a year [5]. Meanwhile, *B. subtilis* is a bacterium that causes food spoilage, such as ropiness in bread [6]. *Bacillus licheniformis* is another spoilage bacterium that forms biofilms quickly in the dairy industry [7].

Both *B. cereus* and *B. subtilis* can form biofilms, a group of bacterial cells living beneath a self-produced matrix of extracellular polymeric substance (EPS). *B. cereus* is a biofilm-forming species with high EPS production and motility [3]. Biofilm matrices are responsible for biofilm adhesion to certain surfaces [8]. They can form on biotic or abiotic surfaces [9]. Biofilms optimize conditions for bacterial survival in food processing. If biofilms form on food contact surfaces, the bacteria sheltered in biofilms can easily contaminate food products. This increases food safety concerns, such as pathogen transmissions that lead to foodborne illnesses and spoilage bacteria transmissions that reduce shelf life and promote financial losses [10]. Biofilm may form via cell-to-cell communication, known as quorum sensing (QS), in unsuitable conditions. One of the strategies to control biofilms is disrupting the quorum sensing system, referred to as quorum quenching (QQ). Due to their matrices, bacterial cells within biofilms are generally more resistant to external stresses or treatments, such as antibiotics and sanitizers [11].

The use of antibiotics has increased over the years and is not just limited to treating bacterial infections in humans. Antibiotics have been used in agriculture and animal farming, for example, to treat plant infections or promote animal growth. This leads to an increase in antibiotic resistance, rendering it ineffective in treating bacterial infections, including biofilm-related infections [12]. According to Yuan et al. [13], biofilm formation in *Salmonella typhimurium* M3 was promoted in the presence of tetracycline at sub-inhibitory concentrations, highlighting the concerns of low environmental antibiotics concentration on foodborne pathogen biofilm-forming ability. Most bacterial cells populating a biofilm complex are antibiotic-resistant. Additionally, biofilms consist of bacterial cells sheltered under a matrix that acts as a barrier, thus protecting the cells from antibiotics, extreme pH and temperature, high pressure, etc. This calls for alternative approaches to combat biofilms, especially in the food industry, such as antibiofilm agents [14].

Alternative antibiofilm agents are therefore required to combat biofilms. Antibiofilm agents may be extracted from natural sources, including plants. Many plants are hosts to bacterial communities, namely plant-associated bacteria (PAB), which are generally classified into endophytic (within plant tissues), phyllospheric (on plant tissues above ground), and rhizospheric bacteria (soils near plant root). PAB is capable of producing beneficial compounds, such as plant-growth hormones and antimicrobial compounds to resist plant pathogens [15]. According to Vanessa and Waturangi (2021), the extracts of phyllospheric bacteria inhibited and destructed biofilms of *B. cereus* and *B. subtilis* [16].

The aim of this study is to determine and quantify the antibiofilm activity of supernatants from plant-associated bacteria recovered from Ternate, Indonesia against *B. cereus* and *B. subtilis*. PAB were molecularly identified and bioactive contents of the supernatants were identified using GC–MS analysis.

Main text

Methods

Bacterial cultivation

Soil samples were obtained from rhizospheres area in Morotai, Tidore, and Ternate, North Maluku A 500-g soil sample was collected at a depth of ± 5–30 cm around the plant roots. Isolation was conducted using the serial dilution method with 0.85% of sterile saline solution. The dilutions were plated on Nutrient Agar (NA; HiMedia) and Yeast Extract-Malt Extract Agar (ISP 2; HiMedia), then incubated at 30 °C for 24–48 h for NA and 7–14 days for ISP 2. Thirteen PAB isolates were obtained (FT5: butterfly pea rhizosphere; F3A.2: butterfly pea nodule; RC2.2: afo clove rhizosphere; RC3.1: afo clove rhizosphere; M6.2: butterfly pea nodule; RCA8: afo clove rhizosphere; RCA4: afo clove rhizosphere; RCA7: afo clove rhizosphere; PTM3: shameplant nodule; DHG3: butterfly pea rhizosphere; DWR1: butterfly pea rhizosphere; TE1: gold mine soil; TE2: gold mine soil). All PAB isolates were cultured on Luria–Bertani Agar (LA; Oxoid) (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 20 g of agar bacto, 1 L of distilled water) and incubated at 28 °C for 24 h. QS indicator bacterium, wild-type *C. violaceum*, was obtained from Atma Jaya Culture Collection and cultured on LA and incubated at 28 °C for 48 h. *Bacillus cereus* ATCC 10876 and *Bacillus subtilis*

ATCC 6633 were cultured on LA and incubated at 37 °C for 24 h. All isolates were stored at -4 °C and routinely cultured.

Supernatant production

Each PAB isolate was cultured in Luria–Bertani Broth (LB; Oxoid). Cultures were transferred into sterile conical tubes and centrifuged at 5752 × *g* for 20 min. Supernatants were collected and freeze-dried for 48 h, then stored at − 20 °C until further use [17].

Detection of antimicrobial activity

Inhibition of planktonic growth was detected using the agar well diffusion method [18]. *B. cereus* and *B. subtilis* were each cultured in LB and adjusted to OD600 of 0.132. Cultures were continuously streaked onto Mueller–Hinton Agar (MHA; Oxoid). The obtained supernatants were loaded into wells created with cork borers. Plates were incubated at 37 °C for 24 h. Streptomycin 10 mg/mL was used as positive control and 1% dimethyl sulfoxide (DMSO) was used as negative control. This method was performed in triplicates.

Detection of quorum quenching activity

Quorum quenching activity was detected using the agar well diffusion method [18]. Wild-type *C. violaceum* was cultured in LB. The culture (OD600 = 0.132) was continuously streaked onto LA. The obtained supernatants were loaded into wells created with cork borers. Plates were incubated at 28 °C for 24 h. Streptomycin 10 mg/mL was used as positive control and 1% DMSO was used as negative control. This method was performed in triplicates.

Quantification of antibiofilm activity

Antibiofilm activity was quantified using two assays: inhibition and destruction. *B. cereus* and *B. subtilis* were cultured in Brain Heart Infusion Broth (BHIB; Merck) supplemented with 2% glucose (Merck). For inhibition assay, 100 µL of each culture (OD600 = 0.132) was added with 100 µL of each supernatant into a 96-well microplate, then incubated (37 °C, 24 h). For destruction assay, 100 µL of each culture (OD600 = 0.132) was added into a 96-well microplate and incubated (37 °C, 24 h). Then, 100 µL of each supernatant was added and incubated (37 °C, 24 h). Sterile culture media and the cultures of *B. cereus* and *B. subtilis* were used as controls. After incubation, media and cells were discarded. Wells were rinsed and air-dried. Cells were stained with 0.4% crystal violet for 30 min, then the dye was discarded. Wells were rinsed and air-dried, then 96% ethanol was added to each well. Absorbance of the final mixtures was measured at 595 nm (Tecan M200 Pro); 96% ethanol was used as blank. This method was performed in triplicates. The percentage of biofilm inhibition and destruction was calculated using the formula [19]:

$$\text{Inhibition or Destruction (\%)} = \frac{\text{Abs. growth control} - \text{Abs. sample}}{\text{Abs. growth control}} \times 100\%$$

Microscopic observation

Destructed biofilms were observed using light microscopy (LM) and scanning electron microscopy (SEM). Supernatants exhibiting the highest destruction activity to *B. cereus* and *B. subtilis* were selected. *B. cereus* and *B. subtilis* were each cultured in BHIB supplemented with 2% (w/v) glucose. Each culture (OD600 = 0.132) was added onto a sterile cover glass and incubated for 24 h at 37 °C. Each elected supernatant was added onto the cover glass and reincubated overnight. Cover glass was rinsed with distilled water. For LM, cover glass was stained with 0.4% crystal violet for 10 min, rinsed, and observed at 40 × magnification. For SEM, samples were fixed with 2.5% (w/v) glutaraldehyde at 4 °C overnight. Samples were dehydrated with ethanol for 15 min in each concentration: 30% (v/v), 50% (v/v), 70% (v/v), 96% (v/v), and 100% (v/v); followed by a 10-min incubation period at 37 °C to dry samples. Samples were coated with gold and observed using SEM [20].

Toxicity assay

This assay was performed using brine shrimp lethality assay (BSLA). Artificial seawater was prepared by dissolving 38 g of NaCl in 1 L of distilled water. Sample stock solutions of 10,000 µL/L were prepared using artificial seawater as solvents, then diluted to 100, 500, and 1000 µL/L. Three milligrams of brine shrimp eggs were hatched in 350 mL of artificial seawater overnight at room temperature with aeration and illumination. Hatched nauplii were transferred into artificial seawater (4.5 mL) in test tubes. Each PAB supernatant (0.5 mL) was added into the tubes and incubated under the same conditions. Potassium dichromate ($K_2Cr_2O_7$) 10 mg/mL was used as positive control and artificial seawater was used as negative control. The mortality percentage was calculated using the formula [21]:

$$\text{Mortality (\%)} = \frac{\text{Dead nauplii}}{\text{Total nauplii}} \times 100\%$$

Molecular identification of plant-associated bacteria

Plant-associated bacterial DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). DNA amplification used a mixture of 12.5 µL of GoTaq, 9.5 µL

of ddH_2O, 1 µL of DNA template, 1 µL of forward primer 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1 µL of reverse primer 1387R (5′-GGGCGGAWGTGTACAAGGC-3′) [22]. PCR conditions were set to: pre-denaturation (94 °C, 2 min), 25 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), elongation (72 °C, 1 min), post-elongation (72 °C, 20 min) [23]. PCR-amplified products were separated using agarose gel electrophoresis (1% agarose) and visualized using GelDoc. 16S rRNA gene sequencing was conducted by Genetika Science and the results were submitted to GenBank.

GC–MS analysis

Contents of PAB supernatants were analyzed using GC Trace 1310, MS ISQ LT, and TG-5MS. Each of the thirteen PAB supernatants was diluted in 2 mL of sterile LB and filtered with a 0.22 µm microfilter. The samples were each injected at a volume of 1 µL. Helium gas 99.999% was used as carrier gas at 1 mL/min flow rate. The oven was set to 250 °C [23].

Statistical analysis

Data analysis was carried out with IBM SPSS Statistics 25 using parametric independent-samples t-test and non-parametric Mann–Whitney test.

Results

Detection of antimicrobial activity

Among thirteen PAB supernatants, none affected the planktonic growth of *B. cereus*. Four PAB supernatants, namely FT5, F3A.2, RC2.2, and PTM3, formed clear zones around the wells which indicate *B. subtilis* growth inhibition (Supplementary Table 1).

Detection of quorum quenching activity

Eight PAB supernatants, namely FT5, RC3.1, RCA4, RCA7, PTM3, DWR1, TE1, and TE2, generated opaque zones around the wells which indicate QQ activity against wild-type *C. violaceum* (Supplementary Table 2).

Quantification of antibiofilm activity

In this assay, four PAB supernatants which earlier showed antimicrobial activity were excluded from the inhibition assay since any biofilm inhibition may be caused by inhibited growth. Each supernatant showed both inhibition and destruction activities against biofilms of *B. cereus* and *B. subtilis* (Table 1, supplementary Fig. 1 and 2).

Microscope observation

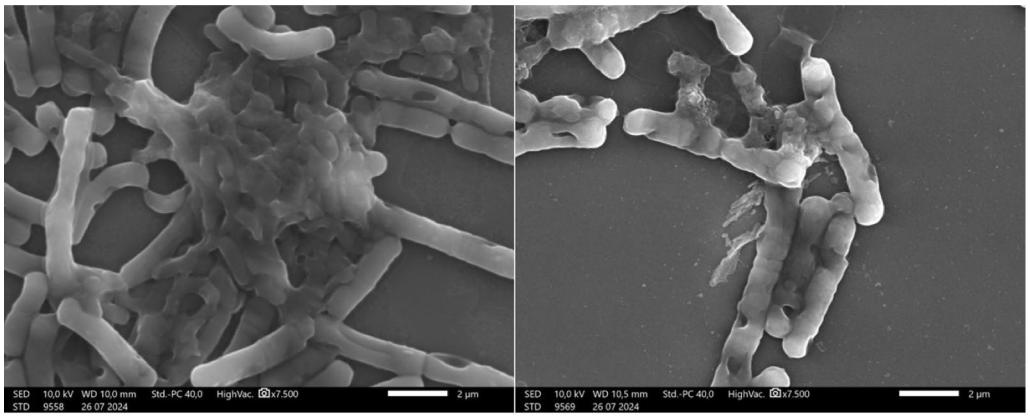
Both LM and SEM showed that biofilms formed by *B. cereus* and *B. subtilis* treated with the supernatants of RCA8 and FT5, respectively, were prominently reduced compared to the control groups (Fig. 1a, b). Based on EDS, *B. cereus* control and treatment groups contained C, N, O, Na, Mg, Si, P, and Ca. *B. subtilis* control group also contained the same elements with the addition of Al, although N and P were not detected in the treatment group (Supplementary Table 3).

Toxicity assay

Supernatants with the highest antibiofilm activities, namely FT5, F3A.2, RCA8, and DHG3, were selected for BSLA. All supernatants at every concentration produced 0% mortality rates.

Table 1 Antibiofilm activity (%) of plant-associated bacteria supernatants against *B. cereus* and *B. subtilis*

Isolates	*B. cereus*		*B. subtilis*	
	Inhibition (%)	Destruction (%)	Inhibition (%)	Destruction (%)
FT5	77.23	71.85	–	81.43
F3A.2	48.01	72.45	–	77.75
RC2.2	74.10	73.16	–	77.23
RC3.1	73.63	69.97	44.25	73.35
M6.2	82.11	70.35	62.13	69.24
RCA8	83.07	77.99	76.12	71.04
RCA4	73.69	77.34	51.88	69.03
RCA7	43.50	36.55	43.24	61.42
PTM3	39.33	75.31	–	59.97
DHG3	84.87	77.38	70.83	76.37
DWR1	81.35	77.11	69.19	66.20
TE1	77.70	76.32	70.35	70.02
TE2	44.03	71.41	62.45	69.71



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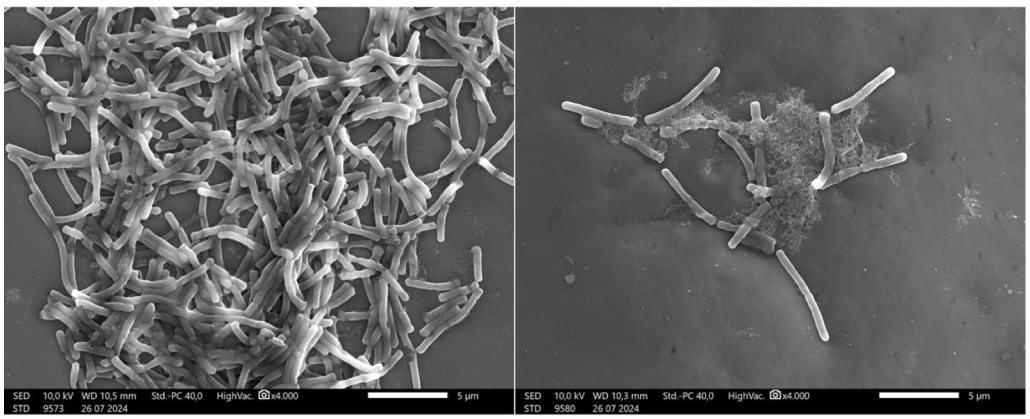

b

Fig. 1 SEM observation of *B. cereus* and *B. subtilis* destructed biofilms. **a** *B. cereus* control (left) and control + RCA8 (right). **b** *B. subtilis* control (left) and control + FT5 (right)

Molecular identification of plant-associated bacteria

16S rRNA gene sequences revealed similarities of PAB isolates to three genera, namely *Bacillus*, *Priestia*, and *Chryseobacterium*. All results have been submitted to GenBank with the assigned accession numbers (Supplementary Table 4).

GC–MS analysis

Contents of bioactive compounds in PAB supernatants exhibiting the highest antibiofilm activities were analyzed using GC–MS (Supplementary Fig. 3–8). Some compounds belong to similar groups and several individual compounds were found in multiple PAB supernatants (Table 2).

Statistical analysis

Independent samples t-test and Mann–Whitney test revealed significant differences ($p < 0.05$) between all control and treatment groups of *B. cereus* and *B. subtilis* in each inhibition and destruction assays (Supplementary Fig. 9–12).

Discussion

One of the challenges in the food industry is food contamination via biofilms, a complex that provides cell resistance to antimicrobial and other harsh treatments [2]. Therefore, alternative antibiofilm agents are required to combat biofilms. In this study, antibiofilm activities of thirteen PAB supernatants were assessed against *B. cereus* and *B. subtilis*. Based on the antimicrobial activity assay, four supernatants which inhibited the planktonic growth of *B. subtilis* contained n-hexadecanoic acid, trans-13-octadecenoic acid, and 2-hydroxy-1-(hydroxymethyl)ethyl ester (Table 2), which inhibited planktonic growth of *B. subtilis* [24].

Table 2 Bioactive compounds in supernatants of plant-associated bacteria

Isolate	Compound name	Area %
FT5	trans-13-Octadecenoic acid	23.41
	n-Hexadecanoic acid	14.41
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	3.80
F3A.2	trans-13-Octadecenoic acid	26.98
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	9.10
	n-Hexadecanoic acid	17.55
RC2.2	trans-13-Octadecenoic acid	23.16
	n-Hexadecanoic acid	16.21
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	3.86
RCA8	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-	4.54
	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	3.61
	n-Hexadecanoic acid	6.04
PTM3	n-Hexadecanoic acid	8.19
	Ergotaman-3′,6′,18-trione,9,10-dihydro-12′-hydroxy-2′-methyl-5′-(phenylmethyl), (5′α,10α)-	3.68
	trans-13-Octadecenoic acid	8.48
DHG3	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-	5.80
	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	4.77
	n-Hexadecanoic acid	7.23

Eight PAB supernatants demonstrated QQ activity against wild-type *C. violaceum* (supplementary Table 2). This bacterium is a model organism in QS-related studies due to its QS-mediated violacein pigment production, observable as purple colonies. Violacein synthesis is regulated by the CviI/CviR QS system [25].

Based on antibiofilm activity quantification, PAB supernatants were able to inhibit biofilm formation and destruct formed biofilms of *B. cereus* and *B. subtilis*. The highest biofilm inhibition and destruction activities against *B. cereus* biofilms were exhibited by DHG3 (84.87%) and RCA8 (77.99%), respectively. Biofilms of *B. subtilis* was best inhibited by RCA8 (76.12%), while the highest destruction activity was shown in FT5 (81.43%) (Supplementary Table 3). Varying percentages are due to different types and amounts of contents in PAB supernatants, which contribute various antibiofilm mechanisms [26]. EPS compositions in *B. cereus* and *B. subtilis* biofilm also affect their capabilities in sustaining biofilm stability [27]. Based on the previous assay, the antibiofilm mechanism of PAB supernatants may be via QS disruption occurring in *B. cereus* and *B. subtilis* which utilize autoinducer peptides (AIPs) [28]. For example, kinase inhibitors can disrupt QS in some Gram-positive bacteria [29].

Enzymes can also disperse biofilms by degrading matrix components, for example polysaccharides, DNA, and proteins, which can be hydrolyzed by glycosidases, deoxyribonucleases, and proteases [32]. LM and SEM showed that both control groups produced dense biofilms which decreased in the treatment groups. *B. subtilis* biofilms contain three major proteins: TapA, TasA, and BslA. TapA attaches fibers formed by TasA to bacterial cell walls to increase biofilm durability, while BslA is a hydrophobic film that surrounds biofilms. In *B. cereus*, fibers are formed by TasA and CalY proteins [33]. Based on EDS, the elements C, N, and P decreased after treatment. These elements account for major biofilm EPS, namely polysaccharides, proteins, and DNA [17]. O, Na, and Si increased after treatment. As biofilm depth increases, the oxygen concentrations inside are reduced [34]. Sodium chloride, containing Na, induced stresses that led to attachment and aggregation in biofilm formation [35]. Mg and Ca also facilitate biofilm initial attachment [36]. The increase of silicone was potentially caused by adsorption from the cover glass in sample preparation [23]. Al was decreased in *B. subtilis* treatment group. Aluminum may decrease after treatment due to its usage for improving biofilm maturation [23].

Based on BSLA, supernatants of FT5, F3A.2, RCA8, and DHG3 had no mortality effect on brine shrimp at 1000 µL/L. The selected supernatants are considered non-toxic since the highest concentration of 1000 µL/L had 0% mortality rates [37].

The 16S rRNA gene sequences of thirteen PAB isolates resulted in > 98% identity to the genera of *Bacillus*, *Priestia*, and *Chryseobacterium*. Extracts of *Priestia aryabhattai* and *Bacillus* spp. were reported to inhibit biofilms of *B. subtilis* and *Staphylococcus aureus*, respectively [38, 39]. *Priestia megaterium* and

Chryseobacterium sp. produced cellulase that disrupted *B. cereus* biofilms which require the presence of cellulose [30, 31].

GC–MS analysis showed an abundance of fatty acids: n-hexadecanoic acid, known to inhibit biofilm formation in *Bacillus spizizenii* and *S. aureus* by impeding cell adhesion to surfaces [40], and trans-13-Octadecenoic acid, which inhibits *S. aureus* biofilms by disrupting QS regulator genes [41]. Some supernatants contained ethyl esters which interacted with QS receptors in wild-type *C. violaceum* to lower EPS production [42]. Pyrrolo[1,2-α] pyrazine-1,4-diones were found in several supernatants, which inhibited biofilm formation and disrupted formed biofilms in *Escherichia coli* [43, 44].

Limitations

Further studies should be conducted to determine the molecular antibiofilm mechanism of the compounds contained in PAB supernatants. Toxicity assays should be performed on other subjects considering future applications to human-consumed food products.

Abbreviations

EPS	Extracellular polymeric substance
QS	Quorum sensing
QQ	Quorum quenching
PAB	Plant-associated bacteria
LM	Light microscopy
SEM	Scanning electron microscopy
BSLA	Brine shrimp lethality assay

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06997-0.

Additional file 1.

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Author contributions

GG conducted the research, data analysis, and wrote the manuscript under the advisory of DEW. DEW designed the study, revised the manuscript, data analysis, and supervised the whole research. NP and N: conducted bacterial isolation and preservation. PGSJ: data analysis. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The 16S rRNA gene sequences have been deposited into GenBank for PAB isolates: FT5 (Accession Number: PP893326), F3A.2 (PP894785), RC2.2 (PP894788), RC3.1 (PP894161), M6.2 (PP894818), RCA8 (PP894162), RCA4 (PP894825), RCA7 (PP894831), PTM3 (PP894163), DHG3 (PP894833), DWR1 (PP894183), TE1 (PP894923), and TE2 (PP894202).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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