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Quantification of anti-quorum sensing and antibiofilm activity of phyllosphere bacteria against food spoilage bacteria

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Abstract

Food spoilage and microbial contamination require attention during the food production process since the presence of these bacteria can create problems including the formation of biofilms produced by these bacteria. Biofilm formations are initiated through cell-to-cell communication which is called quorum sensing mechanism. Hence, inhibition of this communication mechanism could be one of the solutions to inhibit biofilm formation. Therefore, exploration of bioactive compounds from various sources including phyllosphere bacteria with anti-quorum sensing inhibition activities is important. Phyllosphere bacteria are a community of bacteria found on the surface of plant leaves at a very large population. These bacteria can produce bioactive compounds that can inhibit quorum sensing mechanism. In this study, 54 phyllosphere bacteria isolates were tested, 8 bacterial isolates had potential effect to inhibit quorum sensing. From biofilm inhibition assay, the highest percentages were showed by different phyllosphere isolates against each pathogen. Whereas, for biofilm destruction assay, JB 8F isolate had the highest percentage of destruction biofilm activity against biofilm formed by *Bacillus cereus* and *Shewanella putrefaciens*. Eight isolates of phyllosphere bacteria had the potential as quorum quencher and anti-biofilm agents, both for inhibition and destruction of biofilm.

Introduction

Food quality and safety must be considered when making food products. This lack of attention can cause food contamination and spoilage, causing illness in people. Food spoilage is caused by pathogenic bacteria that have the ability to make biofilms. Biofilms form bacterial communities that attach to the food surface and produce extracellular polymeric substances (EPS). Biofilms can be found on food or on the surface of food production equipment that causing food spoilage (Zhang *et al.* 2009). Bacteria that can cause food spoilage include *Bacillus cereus*, *Bacillus subtilis*, and *Shewanella putrefaciens*. Some strains of *B. cereus* could contaminate the food production equipment pipelines by forming biofilms. This bacteria can cause food poisoning by producing enterotoxins and emetic toxins (Lindsay *et al.* 2006; Voort and Abee 2009). *Bacillus subtilis* can produce biofilms when hot fluids continuously flow over a surface and contaminate dairy products (Lindsay *et al.* 2006). Meanwhile, *S. putrefaciens* biofilm can cause spoilage in fish products and other aquatic food products (Bai and Rai 2011). Besides that, *S. putrefaciens* can also cause food poisoning by production of neurotoxins, called tetrodotoxin

(TTX) (Wang *et al.* 2013).

Most of biofilms production is initiated through quorum sensing mechanism, where quorum sensing is a communication mechanism between bacteria and regulates gene expression of extracellular molecular signals (Hadiwiyono 2009). To overcome this problem, it is important to inhibit bacterial communication. Quorum sensing inhibition can be done by degrading extracellular signal molecules using enzymes. One of the bacteria that can produce metabolites which can interfere with quorum sensing process is phyllosphere bacteria. These bacteria are a group of bacteria living on the surface of the plant leaves that have a very large population and have not been much explored. Phyllosphere bacteria can produce bioactive compounds that can inhibit the process of quorum sensing. For example, this bacteria can fight phytopathogenic *Dickeya dadantii* by inhibiting of quorum sensing through production of AHL-lactonase as quorum quencher (Satwika *et al.* 2017). The new discoveries can be made regarding phyllosphere bacteria as an anti-biofilm that can be done by interfering quorum sensing or destruction of biofilms that have been formed.

The aim of this research was to screen anti-quorum sensing activity and quantification of anti-biofilm activity of phyllosphere bacterial isolates against food spoilage bacteria, such as *B. cereus*, *B. subtilis*, and *S. putrefaciens*.

Experimental

Phyllosphere bacterial isolates

Fifty-four phyllosphere bacterial isolates were recovered from the Atma Jaya Culture Collection (Catholic University of Indonesia, Jakarta). These bacteria were isolated during previous study and were recovered from *Psidium guajava*, *Averrhoa carambola*, and *Anredera cordifolia* leaves (Juliana 2011; Listriani 2011). For isolation of phyllosphere bacteria, the leaves were put into the tubes containing 10 mL of 10 mM of phosphate buffer (pH 8.0). Then, the tubes were put into a sonicator for 5 min to release the bacteria from the leaves and homogenized in the buffer. After that, the bacteria suspensions were diluted to phosphate buffer with different serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). A total of 100 µL of the bacterial suspensions were spread on the Brain Heart Infusion Agar (BHIA) (Oxoid Ltd., Basingstoke, United Kingdom) and incubated 28 °C for 48 h. Phyllosphere bacterial isolates from previous study were grown into King's B Agar (2 g protease peptone; 1.5 mL glycerol; 0.15 g K_2HPO_4; 0.15 g $MgSO_4 \cdot 7H_2O$; 20 g bacteriological agar; 1 L distilled water) and incubated at 28 °C for 48 h. King's B is a semi-selective media for phyllosphere bacteria containing glycerol as an abundant carbon source (Humphrey *et al*. 2014). In this study, the majority of phyllosphere bacterial isolates were recovered from *Psidium guajava*. From the previous study these bacteria showed rod-shape and Gram-negativity. Biochemical properties for all the isolates showed as *Proteus* sp. Molecular identification was done only for JB 16B that was identified as *Proteus myxofacies* with accession number HQ852045 (Juliana 2011).

Food spoilage bacteria

The food spoilage bacteria used in this study were *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, and *Shewanella putrefaciens* ATCC 8071. *B. cereus* and *B. subtilis* were inoculated on Luria Agar (5 g NaCl; 5 g yeast extract; 10 g tryptone; 20 g bacteriological agar; 1 L distilled water) (LA) and incubated overnight at 37 °C. *S. putrefaciens* were inoculated on Tryptone Soya Agar (TSA) (Oxoid Ltd., UK) and incubated overnight at 28 °C.

Screening for anti-quorum sensing activity

Screening of anti-quorum sensing activity was done using the overlay agar method described by Abudoleh and Mahasneh (2017) with some modification. Phyllosphere bacteria isolates were inoculated on King's B media by the straight streak method and then incubated at 28 °C for 48 h. Indicator bacteria *Chromobacterium violaceum* was inoculated on Luria Broth (LB) (5 g NaCl; 5 g yeast extract; 10 g tryptone; 1 L distilled water) and incubated at 28 °C for 24 h in an orbital shaker. A total of 100 µL of indicator bacteria from culture (OD_{600} = 0.132) were prepared into 7 mL of semisolid Luria Agar (0.75 % agar) and poured to

overlay on the phyllosphere isolate. Then, the media were incubated at 28 °C for 48 h. The positive result of this anti-quorum sensing screening was determined as a clear zone around the phyllosphere isolate which indicates inhibition of violacein pigment production.

Production of crude extract

Phyllosphere extraction was done by using a liquid-liquid extraction method according to Fitriani *et al.* (2016), with some modifications. Phyllosphere bacteria isolates were inoculated into 200 mL of Luria Broth media and cultivated in the orbital shaker incubator at 28 °C for 48 h, and 120 rpm. Then, the culture of the phyllosphere bacterial isolates were transferred into a conical tube and centrifuged at 13,888 × g for 15 min. The cell-free supernatant was mixed with the same volume of ethyl acetate and kept in the orbital shaker incubator at 28 °C, 120 rpm, overnight. A solvent layer was taken and evaporated using a rotary evaporator and vacuum oven overnight to obtain the crude extract. After that, 1 % of dimethyl sulfoxide (DMSO) were added to obtain a final concentration of stock 20 $mg.mL^{-1}$ (w/v) and stored at -20 °C to maintain the stability of crude extract.

Antibacterial activity assay

The antibacterial activity test was done using 3 food spoilage bacteria (*B. cereus, B. subtilis,* and *S. putrefaciens*) using agar well diffusion method. Three pathogenic bacteria were spread onto Brain Heart Infusion Agar (BHIA) (Oxoid Ltd., Basingstoke, United Kingdom) using a sterile cotton bud. Then, a sterile cork borer was used to make wells. A total of 100 µL of phyllosphere's extract were added into each well. Streptomycin (10 $mg.mL^{-1}$) was used as a positive control, while 1 % of DMSO was used as a negative control. The inoculated media were incubated at 37 °C for 24 h. Antibacterial activity was observed by the formation of a clear zone around the well. This assay was performed in triplicates.

Detection of anti-quorum sensing activity

C. violaceum indicator bacteria were used to determine anti-quorum sensing activity using agar well diffusion method. *C. violaceum* was spread onto BHIA media using a sterile cotton bud. Sterile cork borer was used to make wells. A total of 100 µL of phyllosphere's extract were added into each well. Streptomycin (10 $mg.mL^{-1}$) was used as a positive control, while 1 % of DMSO was used as a negative control. Then, the inoculated plates were incubated at 28 °C for 24 h. Anti-quorum sensing activity was observed with the formation of clear zones due to the inhibition of violacein pigment production. This assay was performed in triplicates.

Quantification of antibiofilm activity

Extracts of phyllosphere bacterial isolates were tested against biofilm formation produced by food spoilage bacteria using 96-well microtiter plates. Extracts JB 3B, JB 16B, JB 17B, JB 20B, JB 26B, JB 8F, JB 12F, and EJB 5F were used for this assay. Then, three pathogenic bacteria were grown onto Brain Heart Infusion Broth (BHIB) (Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37 °C for 24 h. For the inhibition assay, 100 µL of each food spoilage bacteria culture (OD_{600} = 0.132) and 100 µL of extract of each phyllosphere were put into 96-well microtiter plates and incubated at 37 °C for 24 h. While for the destruction assay, 100 µL of each pathogenic bacteria culture (OD_{600} = 0.132) were put into 96-well microtiter plates and incubated at 37 °C for 24 h. After incubation, 100 µL of the extract of the phyllosphere were put into 96-well microtiter plates and re-incubated at 37 °C for 24 h. Then, all of the media were removed, and the adherent cells were rinsed twice by distilled water. Cells that had been rinsed were air dried before staining. Biofilms were stained with 200 µL of 0.4 % (w/v) of crystal violet solution for 30 min. The dye was removed, and the wells were rinsed 5 times by distilled water and air-dried for 5 min. The last step was the addition of 200 µL of ethanol into each well and resuspension to solubilize the crystal violet. Optical density was determined using a microplate reader at a wavelength of 595 nm and the percentage of inhibition or destruction was calculated by the formula (Eq. 1):

$$\% \text{ inhibition or destruction} = \frac{(\text{Control OD595nm-Test OD595nm})}{\text{Control OD595 nm}} \times 100\ \% \quad (1)$$

Results

Screening for anti-quorum sensing activity

Screening for anti-quorum sensing activity was carried out by overlay agar method. The positive results of this assay were recorded as a clearing zone around the phyllosphere isolates as shown in Fig. 1, that indicated the inhibition of violacein pigment production. From this assay, there were 35 out of 54 phyllosphere isolates showing anti-quorum sensing activity.

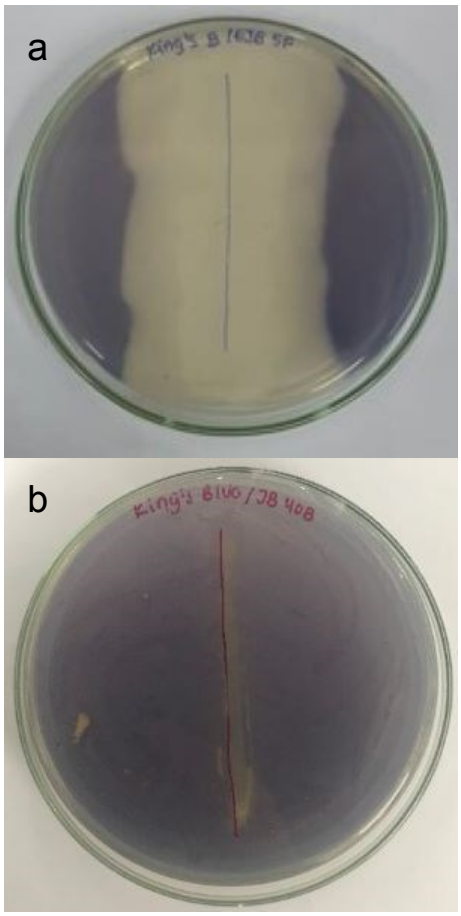

Fig. 1. Screening for anti-quorum sensing activity: positive result from EJB 5F (**a**) and negative result from JB 40B (**b**).

Antibacterial activity assay

Antibacterial activity assays were done using agar well diffusion method. Three food spoilage bacteria were used, including *B. cereus, B. subtilis,* and *S. putrefaciens*. The results revealed that the formation of a clearing zone was produced around the well (Fig. 2). It was performed that there were 4 (JB 20B, JB 22B, JB 40B, JB 12F) out of 54 phyllosphere isolates with antibacterial activity against *B. cereus*, while 2 isolates (JB 16B and JB 12F) showed an antibacterial activity against *B. subtilis*.

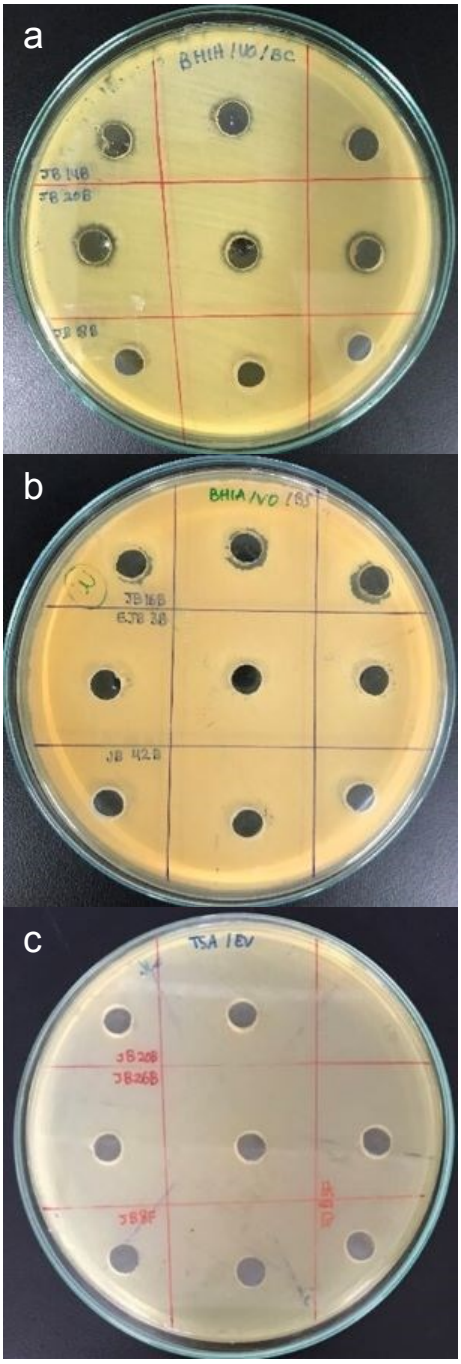

Fig. 2. Antibacterial activity assays of phyllosphere bacteria against *Bacillus cereus* (**a**), *Bacillus subtilis* (**b**), and *Shewanella putrefaciens* (**c**).

Detection of anti-quorum sensing activity

The crude extracts of 8 out of 54 phyllosphere bacterial isolates gave positive results. Anti-quorum sensing activity was determined as clearing zones around the well (Fig. 3). The isolates that had positive results were JB 3B, JB 16B, JB 17B, JB 20B, JB 26B, JB 5F, JB 12F, and EJB 5F.

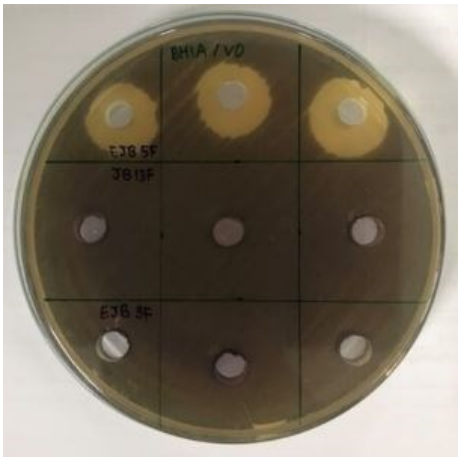

Fig. 3. Detection of anti-quorum sensing activity.

Quantification of antibiofilm activity

The crude extracts of phyllosphere bacteria had the ability to produce antibiofilm against biofilm formed by food spoilage bacteria, such as *B. cereus*, *B. subtilis*, and *S. putrefaciens,* either through inhibition or destruction of the mature biofilm formed. All the crude extracts of the phyllosphere could inhibit the formation of biofilms with a value of mean percentage >50 %. For biofilm of *B. cereus*, 6 crude extracts were tested and the highest inhibition against biofilm of *B. cereus* was 85 % for JB 17B isolate. The lowest inhibition against biofilm of *B. cereus* was 59 % for JB 16B isolate. For quantification of antibiofilm against biofilm of *B. subtilis,* 6 crude extracts were tested. The highest inhibition was 76 % for JB 20B isolates, whereas the lowest inhibition was 38 % for JB 3B isolate. The highest inhibition against biofilm of *S. putrefaciens* was 94 % for JB 8F isolate and the lowest was 27 % for EJB 5F isolate (Table 1).

All the phyllosphere isolates have the ability to destruct the formation of biofilm. According to the results of biofilm destruction, a value of the mean percentage was >40 %. The highest destruction against biofilm of *B. cereus* was 66 % for JB 8F isolate and the lowest was 37 % for JB 3B isolate. The highest destruction against biofilm of *B. subtilis* was 93 % for JB 17B. The lowest destruction against biofilm of *B. subtilis* was 66 % for JB 3B isolate. While the highest destruction activity against biofilm of *S. putrefaciens* was 83 % for JB 8F isolate and the lowest was 39 % for JB 12F isolate (Table 1).

Table 1. Biofilm inhibition and destruction against food spoilage bacteria.

Extracts	*B. cereus*		*B. subtilis*		*S. putrefaciens*	
	% inhibition	% destruction	% inhibition	% destruction	% inhibition	% destruction
JB3B	69	37	38	66	51	66
JB16B	59	43	-	-	65	80
JB17B	85	45	74	93	93	78
JB20B	-	-	76	75	47	82
JB26B	77	46	56	76	50	66
JB8F	78	66	75	92	94	83
JB12F	-	-	-	-	36	39
EJB5F	75	30	54	89	27	44

- = Not be tested because contains antibacterial activity.

Discussion

Phyllosphere bacteria is one of the largest microbes habitats on the surfaces of plants. These bacterial communities might be beneficial to plants by producing plant growth hormones as well as production of other bioactive compounds (Kim *et al.* 2012). These bioactive compounds are also capable acting as quorum quencher. Our results revealed that during screening of anti-quorum sensing activity, 35 of phyllosphere bacterial isolates out of 54 isolates showed positive results,

where the clearing zones were observed around wells of phyllosphere bacteria. It was indicated as inhibition of violacein pigment production through quorum sensing inhibition of *C. violaceum*. Mechanism of quorum sensing inhibition might be happened through destruction of acyl-homoserine lactone (AHL) molecule which plays an important factor as the autoinducer (Abudoleh and Mahasneh 2017). The ability of phyllosphere bacteria to inhibit quorum sensing might act as a survival strategy for competition in the extreme and stressful environment due to the fluctuations in physical condition and limited nutrients. Phyllosphere bacteria have AHL-degrading activity, such as AHL-lactonase and AHL-acylase. In a previous study phyllosphere bacteria was proven as a potential biocontrol reagent to control plant pathogens such as *E. carotovora* through signal interference using AHL-lactonase (Ma *et al.* 2013).

All of the phyllosphere bacteria in this study were extracted using ethyl acetate and dissolved with DMSO to make the concentration of 20 $mg.mL^{-1}$. Crude extracts of phyllosphere were screened for antibacterial assay, detection of quorum sensing, and quantification of antibiofilm. From antibacterial activity assay, positive results were observed by the formation of clearing zones (Younis *et al.* 2015). There were 4 crude extracts that have antibacterial activity against *B. cereus* and 2 crude extracts against *B. subtilis*. The crude extracts that showed antibacterial activity would not be tested for further assay to avoid false positive result in the antibiofilm determination assay.

From detection of anti-quorum sensing activity, there were 8 crude extracts that showed positive results. These positive results were observed by the clearing zones formation which indicated pigment inhibition activity by crude extracts of phyllosphere. *C. violaceum* as an indicator bacteria produces purple pigment, called violacein, the production is initiated by quorum sensing mechanism with AHL acting as an autoinducer (Zahin *et al.* 2010). The main components of violacein production are CviI, AHL, CviR receptor. Violacein is synthesized from tryptophan substrate, this substrate is the product of *vioABCD* operon. CviI synthase converts adenosyl methionine and fatty acids into AHL. In the presence of high concentration of AHL, quorum signal is triggered and AHL binds CviR and control the *vioA* promoter (Stauff and Bassler 2011).

Quorum sensing is not only about pigment production, this mechanism is a way for bacteria to communicate between cells, to produce and respond to extracellular signal molecule at a spesific cell density. Besides pigment production, quorum sensing can be used by bacteria for virulence, bioluminescene, sporulation, and biofilm formation (Hadiwiyono 2009). Biofilm is a bacterial community that attaches to a surface and produces an exopolysaccharide matrix. The first step of biofilm formation is initial attachment stage. On this stage, the bacteria interact and strongly attach. A conditioning film is formed by the adsorption of organic and inorganic nutrients. After this stage, adhered bacteria will produce exopolysaccaride and adhere more firmly. Then, the biofilm will mature and obtain a complex structure of biofilm. In the final stage, cells can release and colonize to other surfaces or return to a planktonic state (Brackman and Coenye 2015). In some bacterial species, the initial attachment stage can use a quorum sensing mechanism. Bacteria use signal molecules when cell densities are high to colonize and stick to surfaces (Zhang *et al.* 2009). Biofilm can be found in the medical field, environment, or food industry. Biofilms in the food industry might be on the food processing equipments or on the food surface. The presence of biofilms on food surfaces can cause spoilage and pathogen microbe contamination. Same as on the food surface, several bacteria are known to form biofilms on the food processing with different material, such as stainless steel (Ciccio *et al.* 2015). The quorum sensing inhibition can be used to inhibit or destruct biofilm that formed by food spoilage bacteria.

According to the quantification of antibiofilm activity, all of the crude extracts of phyllosphere performed antibiofilm activity, both inhibition and destruction. The highest inhibition against biofilm of *B. cereus* was 85 % for JB 17B isolate. *B. cereus* can form biofilms on immersed glass or steel surfaces. The quorum sensing mechanism that induces biofilm formation in *B. cereus* is regulated by *plcR* which is activated by PapR autoinducer

peptide (AIP) (Pomerantsev *et al.* 2009). The highest inhibition againts biofilm of *B. subtilis* was 76 % for JB 20B isolate. *B. subtilis* is an opportunistic pathogen that can contaminate food products by biofilm formation on food processing equipment. The quorum sensing mechanism that induce biofilm is using ComX autoinducer and competence sporulation factor (CSF) (Shank and Kolter 2011). Other food spoilage bacteria that have been used in this study was *S. putrefaciens*. This bacteria can be found in aquatic environments which can form biofilm on the surface of food processing. *S. putrefaciens* can form biofilm on a variety materials, such as stainless steel and glass. Biofilm-promoting factor A (BpfA) is the protein contributing to biofilm formation with AI-2 signaling molecules in quorum sensing mechanism (Cheng *et al.* 2016). We found that the highest inhibition against biofilm of *S. putrefaciens* was 94 % for JB 8F isolate. Based on quantification of biofilm inhibition, all of the crude extracts of phyllosphere had a variety percentage of inhibition. It might have happened due to the different mechanism of biofilm formation from each food spoilage bacteria.

Based on the results of biofilm destruction, the highest biofilm destruction was for JB 8F isolate against biofilm of *B. cereus* and *S. putrefaciens*. The biofilm destruction activity against biofilm of *B. cereus* was 66 % and against biofilm of *S. putrefaciens* was 83 %. The highest biofilm destruction against biofilm of *B. subtilis* was 93 % for JB 17B isolate. These results indicated crude extracts of phyllosphere performed bioactive compound like protease, DNase, and another enzyme that can destruct the component of biofilm and potential for antibiofilm agent (Ma *et al.* 2013). In the other study, these 8 crude extracts of phyllosphere had the ability to inhibit and destruct biofilm formation of fish pathogenic bacteria, such as *Vibrio harveyi*, *Aeromonas hydrophila*, and *Streptococcus agalactiae* (Nathalia and Waturangi 2021). For further study, it is required to characterize the compound from phyllosphere bacteria which have anti-quorum sensing and antibiofilm activity, toxicity assay for phyllosphere extracts, and molecular identification for phyllosphere bacteria for further application.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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