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Inhibition of Quorum Sensing, Biofilm and Motility of *Escherichia coli* by *Staphylococcus warneri* MG1_8 Bioactive Compounds

(Perencatan Penderiaan Kuorum, Biofilem dan Motiliti *Escherichia coli* oleh Sebatian Bioaktif *Staphylococcus warneri* MG1_8)

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ABSTRACT

Quorum sensing (QS) system in bacteria, mediated by a small signaling molecule called an autoinducer (AI), promotes biofilm formation and other virulence factors that contribute to reducing the susceptibility of treatment agents. *Escherichia coli* is considered bacteria contaminated in the food industry with their biofilm-forming ability which allows them to adhere on surfaces. In this study, the cell-free supernatant (CFS) extract of mangrove forest isolated bacteria, *Staphylococcus warneri* MG1_8, was purified and its possible quorum sensing inhibitor (QSI) metabolites were identified. Fraction A was separated from *S. warneri* MG1_8-CFS extract using gel column chromatography and confirmed as a positive fraction by its ability to inhibit bioluminescence in the biosensor strain *Vibrio campbellii* ATCC BAA-1119, showing 76% inhibition. In addition, pyridoxine (PN) was identified as a major compound in the *S. warneri* MG1_8 CFS extract. The minimum inhibitory concentration (MIC) of PN against *E. coli* ATCC 25922 was 50 mg/mL. The PN at 1/4MIC reduced biofilm formation and cell adherence in *E. coli* strains, as observed under scanning electron microscopy (SEM). Moreover, motility and AI-2 signaling also decreased after exposure to PN at sub-MIC levels. Current research demonstrates that PN can inhibit biofilm formation and motility by interfering with AI-2 QS, suggesting it could be considered a novel biocontrol agent to reduce bacterial contamination in food production.

Keywords: Autoinducer-2; biofilm formation; *Escherichia coli*; pyridoxine; quorum sensing inhibitor

ABSTRAK

Sistem penderiaan kuorum (QS) dalam bakteria yang dimediasi oleh molekul isyarat kecil yang dipanggil autoinduser (AI) menggalakkan pembentukan biofilem dan faktor virulensi lain yang menyumbang kepada pengurangan kerentanan agen rawatan. *Escherichia coli* dianggap bakteria tercemar dalam industri makanan dengan keupayaan membentuk biofilem yang membolehkan mereka melekat pada permukaan. Dalam kajian ini, ekstrak supernatan bebas sel (CFS) bakteria pencilan hutan bakau, *Staphylococcus warneri* MG1_8, telah dimurnikan dan metabolit mungkin perencat penderiaan kuorum (QSI) nya telah dikenal pasti. Pecahan A diasingkan daripada ekstrak *S. warneri* MG1_8-CFS menggunakan kromatografi lajur gel dan disahkan sebagai pecahan positif dengan keupayaannya untuk menghalang bioluminesen dalam strain biosensor *Vibrio campbellii* ATCC BAA-1119 yang menunjukkan 76% perencatan. Selain itu, piridoksina (PN) dikenal pasti sebagai sebatian utama dalam ekstrak *S. warneri* MG1_8 CFS. Kepekatan perencatan minimum (MIC) PN terhadap *E. coli* ATCC 25922 ialah 50 mg/mL. PN pada 1/4MIC pembentukan biofilem terturun dan pematuhan sel dalam strain *E. coli,* seperti yang diperhatikan di bawah mikroskop elektron imbasan (SEM). Selain itu, motiliti dan isyarat AI-2 juga berkurang selepas pendedahan kepada PN pada tahap sub-MIC. Penyelidikan ini menunjukkan bahawa PN boleh merencat pembentukan biofilem dan motiliti dengan mengganggu AI-2 QS, mengesyorkan ia boleh dianggap sebagai agen biokawalan baharu untuk mengurangkan pencemaran bakteria dalam pengeluaran makanan.

Kata kunci: Autoinduser-2; *Escherichia coli;* pembentukan biofilem; perencat pengesan kuorum; piridoksina

INTRODUCTION

Food contamination with microorganisms is a major concern for consumers and the food industry which covers a wide range of public health issues causing foodborne illnesses and mortality in humans worldwide (Elbehiry et al. 2023; Tropea 2022). Contamination can occur at various stages of the food chain, including raw materials, food-contact surfaces, and/or food processing lines at the industrial level (Thakali & MacRae 2021). *Escherichia coli* is a serious foodborne microorganism of concern in the food industry due to its ability to form biofilms that allow bacteria to resist and persist in the industry (Abdallah et al. 2014; Galié et al. 2018). Biofilms are bacterial communities covered by a self-produced extracellular polymeric substance and adhered to surfaces (Abebe 2020). Biofilm formation is one of the most virulent factors, further facilitating bacterial resistance and protecting bacteria from treatment agents, making them difficult to eradicate (Liu et al. 2023; Mirghani et al. 2022). Several studies suggest that bacterial communication during biofilm formation is regulated by the quorum sensing (QS) system (Guilhen, Forestier & Balestrino 2017; Munir et al. 2020; Paluch et al. 2020; Preda & Săndulescu 2019; Urvoy et al. 2022).

The QS system is a cell-to-cell communication within or between bacterial species used to coordinate gene expression when a sufficiently high cell density is reached through the sensing of small signaling molecules called autoinducers (AIs) (LaSarre & Federle 2013). QS plays a crucial role in controlling target gene expression, particularly virulence genes, such as those involved in antibiotic resistance, biofilm formation, pigment production, motility, sporulation, bioluminescence, and other virulence factors (Dimitrova, Damyanova & Paunova-Krasteva 2023; Rutherford & Bassler 2012). Among AIs, Gram-negative bacteria primarily use *N*-acyl homoserine lactones (AHLs), while Gram-positive bacteria commonly use autoinducing peptides (AIPs). Another type of AI used by both Gram-positive and Gram-negative bacteria is the *S*-ribosyl-L-homocysteinase (LuxS) protein-based autoinducer-2 (AI-2) system, which, unlike other AIs, has been identified as an interspecies communication signal among bacterial species (Asfour 2018; Lixa et al. 2015; Nahar et al. 2021). The AI-2 system is a significant QS signal in *E. coli*, controlling its virulence factors, particularly biofilm formation. This factor is crucial for the survival and growth of microorganisms in the food industry, leading to food poisoning, shelf-life reduction, and potential foodborne outbreaks (Liu et al. 2023; Peng et al. 2018; Singh et al. 2021). However, antimicrobial or chemical treatment failures remain major issues in eradicating *E. coli* biofilms, as bacteria within biofilms are protected by a matrix of extracellular polymeric substances (EPSs) (Sharma, Misba & Khan 2019). Additionally, proteomic studies have shown that the downregulation of QS-related proteins not only affects cell-to-cell communication but also influences other virulence factors, including biofilm formation and motility in *E. coli* (Ruan et al. 2021; Villa et al. 2012). Therefore, the AI-2 system is considered a potential novel target for inhibiting biofilms and other virulence factors through QS interference (Deep, Chaudhary & Gupta 2011). This strategy is of interest not only in industrial settings but also in medical fields, as it can inhibit QS-related phenotypes without killing bacteria or promoting resistance mechanisms against antimicrobial agents. Thus, interfering with the AI-2 system could contribute to improving food safety strategies (Ahmed et al. 2019; Jiang et al. 2022).

Pyridoxine (PN), a member of the vitamin B6 family, is naturally found in many foods and used as a dietary supplement (McCormick 2014). Vitamin B6 exists in other forms as well, including pyridoxal (PL), pyridoxamine (PM), and their respective phosphate esters-pyridoxal-5′-phosphate (PLP), pyridoxamine-5′-phosphate (PMP), and pyridoxine-5′-phosphate (PNP). These forms commonly serve as precursors for many proteins and enzymes (Parra, Stahl & Hellmann 2018). While vitamin B6 forms are synthesized by plants, yeasts, and bacteria, mammals obtain vitamin B6 through dietary ingestion or microbial sources (Yoshii et al. 2019). In bacteria, vitamin B6 vitamers are synthesized via the *de novo* biosynthetic pathway from deoxy-xylulose 5-phosphate to pyridoxal phosphate (PLP). PLP can be converted to other B6 vitamers, including PN, PL, PMP, and PM, via the salvage pathway. PdxK, PdxP, and PdxY are responsible for the phosphorylation of PNP or PLP to PN or PL, while PL can be converted to PN by PL reductase (PdxI) (Denise et al. 2023; El Qaidi et al. 2013; Richts & Commichau 2021). PLP is the most important bioactive form of vitamin B6, serving as a cofactor for many proteins and enzymes. It has been reported that more than 160 biochemical reactions require vitamin B6 as a cofactor (about 4% of all described activities) (Percudani & Peracchi 2009). Metagenomic analysis has shown that gut microbiota, including *Bacteroides fragilis, Prevotella copri, Bifidobacterium longum, Collinsella aerofaciens*, and *Helicobacter pylori*, possess a vitamin B6 biosynthesis pathway (Magnusdottir et al. 2015). The synthesis of vitamins by gut microorganisms can help maintain and balance the intestinal health of the host (Yoshii et al. 2019). Moreover, vitamin B6 is commercially important as a feed additive, food additive, and in cosmetics and pharmaceuticals (Acevedo-Rocha et al. 2019; Bampidis et al. 2021). PN in its hydrochloride form, is commonly used as a nutritional additive in foods and beverages, such as cereals, soft drinks, dairy products, preserves, and confectionery. The derivative PN form, quaternary ammonium, and bisphosphonium salts of PN, exhibit high biocidal activity against both planktonic and biofilm-embedded opportunistic bacteria, such as Staphylococci (Kayumov et al. 2015). However, the inhibitory effects of PN on QS, other virulence factors, and associated regulatory genes are largely unknown.

Therefore, this study aims to identify natural PN produced by the mangrove bacteria *Staphylococcus warneri* MG1_8 and investigate its anti-QS activity against virulence factors and QS-related protein expression in *E. coli* strains. The goal is to address the issue and provide a suitable alternative strategy using QS inhibitors to combat bacterial contamination in food production, exerting less pressure on bacterial resistance development to the inhibitory agent.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Staphylococcus warneri MG1_8 isolated from mangrove forest sediment, has been previously studied as a quorum sensing inhibitor (QSI)-producing bacterium with the accession number MZ707653. *S. warneri* MG1_8 was cultured in tryptic soy broth (TSB) at 30 °C with shaking at 150 rpm for 24 h. Biofilm-forming *E. coli* isolates 3.1, 3.8, and 5.5 isolated from frozen meats, as well as *E. coli* ATCC 25922, and enteroaggregative *E. coli* (EAEC) PSU 280, were cultured in Luria Bertani (LB) broth (Difco, USA) (Chaichana et al. 2023). QS biosensor strains, *V. campbellii* ATCC BAA-1117 (*luxN*::tn5) and *V. campbellii* ATCC BAA-1119 (*luxM*::tn5) were cultured in marine broth (MB) (Difco, USA) for 18 h at 30 °C with a shaking.

PURIFICATION OF *S. warneri* MG1_8-CFS EXTRACT

Gel column and thin-layer chromatography analyses were applied to separate and purify compounds in *S. warneri* MG1_8-CFS extract. The extraction processes were described in our previous study (Chaichana et al. 2023). For gel column chromatography, Sephadex LH-20 (Sigma-Aldrich, USA) was used to separate mixture compounds in *S. warneri* MG1_8-CFS extract as previously described with a few modifications (Hu et al. 2022). The extract (200 mg/mL) was carefully transferred onto the surface of the gel bead. After elution with 300 mL of absolute methanol, 3 mL of each 28 fractions was collected and analyzed by thin-layer chromatography (TLC). Analytical TLC was performed on TLC Silica gel 60 F_{254} (Merck, Germany). The spot pattern of the TLC chromatogram allowed for the combination of fractions 15-22 as fraction A and fractions 23-27 as fraction B. Fractions A and B were subsequently determined for the bioluminescence inhibition against biosensor strain.

ANTI-QS ACTIVITY OF GEL COLUMN CHROMATOGRAPHY FRACTIONS

The confirmation of gel column chromatography fractions (fractions A and B) for anti-QS activity against *V. campbellii* ATCC BAA-1119 (AI-2 producer) was performed in 96-well white bottom microtiter plates (Greiner Bio-one, Austria) as previously described (Ryu et al. 2016). The biosensor strain was cultured and adjusted to 0.5 McFarland in MB. The 20 µL of bacterial culture and 20 µL of each fraction were added to wells containing 160 µL of MB. Each fraction was prepared in a concentration ranging from 100 to 6.25 mg/mL for testing. The plate was incubated, and bioluminescence production was evaluated at time intervals of 0, 3, 6, and 12 h at 30 °C using a LUMIstar Omega microplate reader (BMG LabTech, Germany). The experiment was carried out in triplicate.

IDENTIFICATION OF QSI ACTIVE COMPOUNDS IN FRACTION A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

Metabolite profiling in fraction A of *S. warneri* MG1_8 extract was performed using LC-QTOF-MS/MS instrument (1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, USA). A sample was dissolved with 2 mL of HPLC-grade methanol followed by filtering via 0.22 µm nylon membrane syringe filters. The LC-QTOF-MS/MS was employed for chromatographic separation through a Zorbax Eclipse Plus C18 Rapid Resolution HD column (150 mm length × 2.1 mm inner diameter, 1.8 µm particle size) maintained at 45 °C. The analytical run duration was set to 55 min and the flow profile of the mobile phase is detailed in Table 1, with a pressure of 800 bars. The injection volume was 2 µL. The mass spectrometer was operated in full scan mode (100-1200 m/z). Qualitative identification of compounds was performed by comparing data with records from the METLIN database (Tapfuma et al. 2019).

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

To confirm the presence of PN as the active compound, the active fraction was analyzed by NMR. ^{1}H NMR spectra of the compound were recorded on a 300 MHz Bruker FTNMR Ultra Shield spectrometer using tetramethylsilane as an internal standard. The structure of the compound in fraction A was compared to the PN standard (Sigma-Aldrich, USA).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC value of PN against *E. coli* ATCC 25922 was determined in Muller Hinton Broth (MHB) using the standard broth microdilution techniques according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for antibiotic testing (CLSI 2020). The PN solution was prepared with two-fold serial dilutions ranging from 6.25, 12.5, 25, 50, 100, and 200 mg/mL as the final concentration. The *E. coli* culture was adjusted to the final concentration of 10^5 CFU/mL. The 100 µL of *E. coli* inoculum was cultured with 100 µL of PN at different concentrations at 37 °C for 24 h. The resazurin

was added into each well and incubated for 3 h to evaluate the color change. The non-fluorescent resazurin (blue color) is reduced by active bacteria to the pink color of the fluorescent resorufin.

EFFECT OF PN ON *E. coli* GROWTH

The effect of PN on *E. coli* growth was evaluated in 96-well microtiter plates using the broth microdilution method. PN was tested at final concentrations of MIC, 1/2MIC, and 1/4MIC, with a control containing only medium. The *E. coli* cultures were adjusted to 10^6 CFU/mL and 100 µL of these cultures were added to wells containing 100 µL of each concentration. The plate was incubated at 37 °C for 24 h and kinetic growth was measured every hour at OD 600 nm using LUMIstar Omega microplate reader (BMG LabTech, Germany).

EFFECT OF PN ON *E. coli* BIOFILM

Based on the observed effects of PN on *E. coli* growth, concentrations of 1/4MIC and 1/8MIC were selected for biofilm inhibition assays. The experiment was conducted by a crystal violet (CV) assay in a 96-well polystyrene microtiter plate. Biofilm-forming *E. coli* strains were cultured overnight and then diluted to 10^6 CFU/mL in freshly prepared M9 salts minimal (MSM) medium supplemented with 0.4% glucose for their biofilm-forming conditions. A volume of 100 µL of the *E. coli* suspension was added to wells containing the sub-MIC concentrations (1/4MIC or 1/8MIC) of the PN. The plates were then incubated at 37 °C for 48 h to allow biofilm formation. Bacterial growth was assessed by measuring cell turbidity at OD 600 nm using a microtiter plate reader (BMG LabTech, Germany). For biofilm quantification via the crystal violet (CV) assay, the culture broth was discarded, and each well was gently rinsed with 150 µL of distilled water to remove planktonic cells. The plate was then air-dried for 20 min before adding 200 µL of a 0.1% (w/v) crystal violet solution to each well, allowing the stain to incubate for 30 min. Afterward, the wells were rinsed four times with distilled water to remove excess dye and left to dry for 1 h. The crystal violet stain was then solubilized by adding 200 µL of absolute ethanol to each well. The absorbance at 570 nm was measured using a microplate reader (BMG LabTech, Germany). Each experiment was performed in triplicate, and the specific biofilm formation (SBF) was calculated using the following formula: SBF = (OD_{570} (sample) - OD_{570} (blank)) / (OD_{600} (sample) – OD_{600} (blank)) (Zhang et al. 2014).

Moreover, scanning electron microscopy (SEM) was performed according to Al-Shabib et al. (2017) to observe the biofilm reduction by sub-MIC levels of PN on *E. coli* isolates. The *E. coli* isolates were cultured on glass coverslips (1.0 × 1.0 cm^2) in the presence or absence of 1/4MIC of PN in 24 well polystyrene microtiter plates at 37 °C for 48 h to allow biofilm formation. The bacterial culture was discarded, and the glass coverslips were gently washed twice with phosphate-buffered saline (PBS) and distilled water (DW), and subsequently fixed with 2.5% (v/v) glutaraldehyde at 4 °C for 2 h. The glass coverslips were then washed twice with PBS and DW, and dehydrated with increasing concentrations of ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%) for 15 min each. After drying, the samples were coated with gold and examined with an SEM (Quanta 400, Thermo Fisher Scientific, USA) at × 3000 and × 10000 magnifications.

SWIMMING MOTILITY INHIBITION ASSAY

The effect of PN on the swimming motility of biofilm-forming *E. coli* isolates was evaluated as previously described (García-Heredia et al. 2016). Swimming motility was assayed using LB broth supplemented with 0.3% agar plate containing 1% tryptone and 0.25% NaCl. PN was added to the final concentrations of 1/4 and 1/8MIC or without (control) in swimming motility agar. *E. coli* cultured were diluted at 10^6 CFU/mL and 0.5 µL were placed on the center of the motility plate. The plate was incubated at 37 °C for 16 h, and the swimming motility area was measured by comparing the diameter of the motility zone to that of the control plate.

DETERMINATION OF AI-2 INTERFERENCE OF PN

The AI-2 activity in *E. coli* strains was measured as described previously, with a few modifications (Nahar et al. 2021). In this experiment, *V. campbellii* ATCC BAA-1119 served as a positive control as an AI-2 producer strain, and *V. campbellii* ATCC BAA-1117 acted as the reporter strain to detect exogenous AI-2. The overnight culture of reporter strain *V. campbellii* ATCC BAA-1117 was diluted 1:5000 in a fresh autoinducer bioassay (AB) medium. The 180 µL of the diluted reporter culture was added to a 96-well white-bottom microtiter plate containing 20 µL of each CFS of *E. coli* isolates cultured with or without 1/4MIC of PN. Bioluminescence was qualified as a relative light unit (RLUs) at 5 h with the LUMIstar Omega microplate reader (BMG LabTech, Germany).

STATISTICAL ANALYSIS

The statistical significance was determined by one-way ANOVA using SPSS (version 26) of Windows statistic software (SPSS Inc., USA); a difference was considered a statistically significant result at P-values < 0.05. All data were expressed as mean ± standard deviation (SD) from triplicate samples with two or three independent experiments.

RESULTS AND DISCUSSION

PURIFICATION OF QSI ACTIVE COMPOUND IN *S. warneri* MG1_8-CFS EXTRACT

The mixture of compounds in *S. warneri* MG1_8-CFS extract was separated using gel-column chromatography based on their molecular size. The gel column chromatography fractions obtained from *S. warneri* MG1_8-CFS extract were 27 fractions. Each fraction was subsequently spotted on the TLC silica gel and twice ran with a 7% methanol/chloroform mixture as a mobile phase. The spot pattern detected under UV light showed 2 major intense spots which were then combined into 2 fractions: Fraction A (elutes 15-22) and Fraction B (elutes 23-27), as shown in Figure 1. The TLC spot patterns show the presence of significant compounds in the extract. Additionally, fraction A was particularly intense, indicating that it contains high concentrations of the main component, which should be further identified. Moreover, this study makes a groundbreaking discovery by being the first study to purify and identify metabolites from the CFS extract of marine *S. warneri* MG1_8 that influence AI-2 QS interference in *E. coli*.

GEL COLUMN CHROMATOGRAPHY FRACTIONS ON BIOLUMINESCENCE INHIBITION

The active fraction was confirmed using bioluminescence disruption of the biosensor strain *V. campbellii* ATCC BAA-1119 which is controlled by AI-2 type of QS. The results found that a concentration of 25 mg/mL of the active fraction did not interfere with the biosensor growth while it was presented to reduce bioluminescence. As shown in Figure 2, both fractions have the ability to reduce the bioluminescence production of biosensor strain, with 32% and 44% inhibition at 3 h, respectively. At 6 and 12 h, fraction A displayed the most effective inhibitions, with 65% and 76% bioluminescence reduction, respectively.

Many foodborne bacteria use AI-2 signaling to control their behaviors, especially biofilm formation which allows bacteria to adhere to material surfaces in the food industry (Bai & Rai 2011; Lee, Kim & Lim 2021). Recent findings have shown some natural compounds can effectively disrupt bacterial QS and are emerging as potential AI-2 QS inhibitors. Our previous study investigated a QSI active compound produced by mangrove forest bacteria, *S. warneri* MG1_8-CFS, and its crude extract exhibited AI-2-related phenotypical inhibition including effects on biofilm formation, motility, and AI-2 signaling production against biofilm-forming *E. coli* strains (Chaichana et al. 2023). The anti-QS activity of *S. warneri* MG1_8-CFS crude extract may be attributed to various active compounds secreted into culture supernatants. These compounds are generally primary and secondary metabolites produced during the stationary phase of growth and are involved in numerous beneficial bacterial activities, such as antibiotic production used against other bacteria or fungi, metal transporting agents, hormone-like compounds, or enzymes that facilitate bacterial growth (Demain & Fang 2000; Huang & Tang 2007; Joyce, Lango & Clarke 2011). The findings align with previous research on the role of AI-2 inhibitors in QS interference. For instance, Panayi et al. (2022) demonstrated that ethanolic extracts from aromatic plants showed significant AI-2 inhibition in *E. coli* MG1655 cultures. Compared to our study, the natural extract effectively modulated AI-2 activities in *E. coli* strains. In this study, the fraction A from *S. warneri* MG1_8-CFS purified by gel column chromatography was confirmed as the active fraction, effectively interfering with the bioluminescence production of *V. campbellii* ATCC BAA-1119 at a concentration of 25 mg/mL without affecting biosensor growth. The results indicate that fraction A is the active fraction, potentially containing the AI-2 inhibitor. Moreover, the interference was specific to bioluminescence, suggesting was not due to general toxicity. Therefore, fraction A was selected for further identification of active compounds using LC-QTOF-MS/MS.

IDENTIFICATION OF QSI ACTIVE COMPOUND IN FRACTION A

The chromatographic profile of fraction A was analyzed through LC-QTOF-MS/MS (Figure 3(A)). It was observed that the 6 different peaks were presented at different retention times, with the identified compounds for each peak listed in Table 2. Each compound was identified by comparing its mass spectra to the METLIN database, achieving ≥95% similarity. Interestingly, the highest peak (peak 1) at the retention time of 1.978 min corresponded to pyridoxine (PN) which is the major compound in this fraction. The PN structure obtained from the database is shown in Figure 3(B). The NMR spectrum of fraction A was compared with the PN standard, as shown in Figure 3(C) and 3(D). The NMR spectrum patterns of both samples displayed similar peak signals of functional groups and the PN structure identified by LC-QTOF-MS/MS is depicted in Figure 3(C). Hence, our result confirms that the significant compound present in the *S. warneri* MG1_8-CFS extract is PN. This study represents the first report of *S. warneri* strain MG1_8 isolated from mangrove sediments, demonstrating the production of PN as a major compound that can reduce the bioluminescence signal of the biosensor strain by AI-2 signaling interference. To confirm this finding, the anti-QS activity of PN on *E. coli* QS phenotypical regulation was examined.

ANTI-BACTERIAL ACTIVITY OF PN

The MIC was applied to assess the antibacterial activity of PN through the broth microdilution method. The

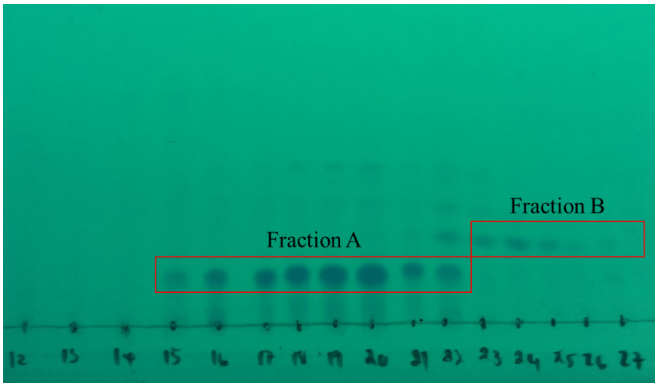

FIGURE 1. TLC profile of *S. warneri* MG1_8-CFS extract with different gel column chromatographic fractions. Eluates 15-22 and 23-27 were pooled and designated as fractions A and B, respectively

TABLE 1. Gradient flow profiles of the mobile phase

Time (min)	Flow rate (mL/min)	Solvent A (water+0.1% formic acid)	Solvent B (methanol+0.1% formic acid)
0	0.200	90%	10%
25	0.200	60%	40%
35	0.200	50%	50%
45	0.200	0%	100%
50	0.200	90%	10%
55	0.200	90%	10%

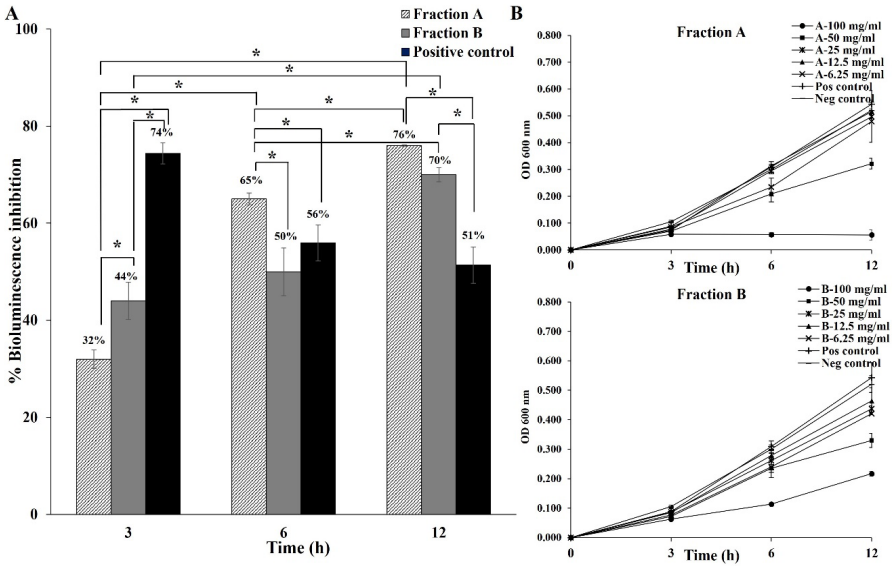

FIGURE 2. The bioluminescence inhibition of gel column chromatography fractions (fractions A and B) at 25 mg/mL concentration and 0.01 mg/mL pyrogallol (positive control) (A) and growth curve of biosensor strain *V. campbellii* ATCC BAA-1119 cultured with fraction A or B with different concentrations (B). Significant differences (Tukey test, $P < 0.05$) compared to other groups

TABLE 2. Identification of major compounds in fraction A of *S. warneri* MG1_8-CFS extract using LC/MS data

Peak	Putative identification	Empirical formula	Molecular mass	Retention time (Rt)	Score
1	Pyridoxine (Vitamin B6)	$C_8H_{11}NO_3$	169.0781	1.978	90.78
2	Enicoflavine	$C_{10}H_{13}NO_4$	211.0847	4.734	49.27
	4-Hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one	$C_{10}H_{11}NO_3$	193.0741	4.851	49.39
	Cyclo-Ala-Pro diketopiperazine	$C_8H_{12}N_2O_2$	168.0898	5.653	48.16
3	1-(2,3-Dihydro-1H-pyrrolizin-5-yl)-1,4-pentanedione	$C_{12}H_{15}NO_2$	205.1103	18.405	49.35
	Sinapoylputrescine	$C_{15}H_{22}N_2O_4$	294.1576	18.497	47.44
4	N-Methylanthranilic Acid	$C_8H_9NO_2$	151.0635	19.023	89.6
	L,L-Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	210.1373	19.195	48.58
	(4-Hydroxybenzoyl)choline	$C_{12}H_{18}NO_3$	224.1289	19.633	49.59
5	3,3'-Dimethoxybenzidine	$C_{14}H_{16}N_2O_2$	244.1219	22.056	48.6
6	4-(N-Maleimido) benzyltrimethylammonium	$C_{14}H_{17}N_2O_2$	245.1297	23.422	47.95

Identification was compared with the METLIN database with ≥95% similarity.
Peak numbers and retention times (Rt) refer to LC/MS chromatograms in Figure 3(A)

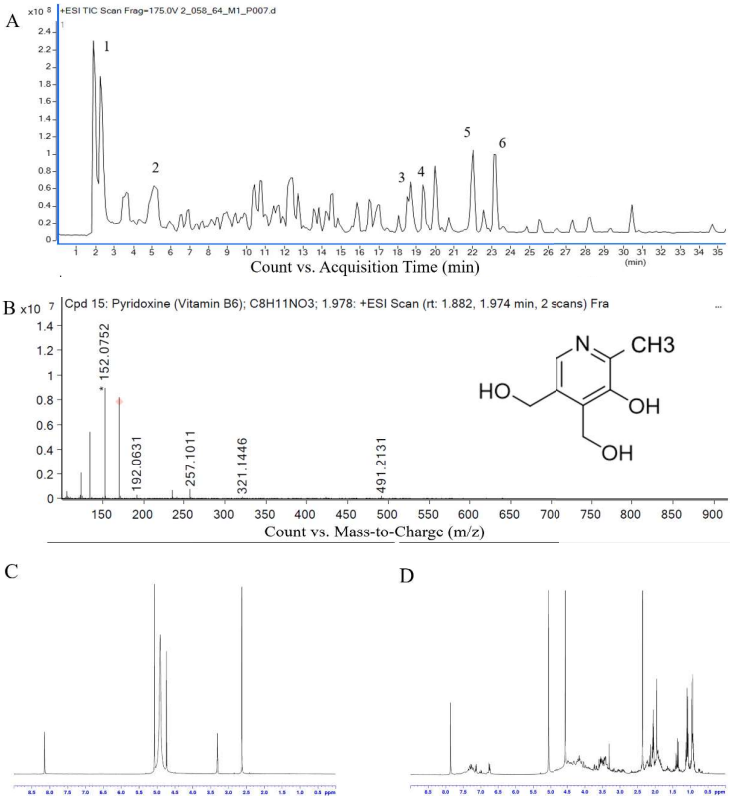

FIGURE 3. The identification of metabolites in fraction A. LC/MS chromatograms of possible compounds in fraction A of *S. warneri* MG1_8-CFS extract (A), PN structure obtained from METLIN database (B), NMR results of PN standard (C) and fraction A sample (D)

cumulative MIC concentration of PN against standard strain *E. coli* ATCC 25922 was found to be 50 mg/mL. Consequently, sub-MIC concentrations (1/4 and 1/8MIC) were used to determine anti-QS activity. In this study, PN exhibited a high MIC value against standard strain *E. coli* ATCC 25922 indicating its limited antibacterial efficacy. However, it is important to note that anti-QS compounds typically do not affect bacterial growth but instead inhibit virulence factors (Zhao, Yu & Ding 2020). PN with a high MIC value aligns with the understanding that effective anti-QS agents can exhibit minimal antibacterial activity while still being effective in modulating bacterial communication and virulence. According to the findings of Ibrahim et al. (2020), they observed that thiazole derivatives with high MICs effectively disrupted QS systems against methicillin-resistant *Staphylococcus aureus* and other multidrug-resistant bacteria without significantly affecting bacterial growth. Therefore, PN is considered suitable for use as an anti-QS agent.

EFFECT OF PN ON THE GROWTH OF *E. coli*

To investigate the effect of PN on the QS system, the growth of biofilm-forming *E. coli* strains in the presence of different PN concentrations was determined. The MIC value of PN against *E. coli* ATCC 25922 was 50 mg/mL. As shown in Figure 4, the growth curve of *E. coli* in the control group entered the log period within 9-12 h of incubation and then reached the stationary phase after 12 h, depending on the strains. In contrast, PN at the MIC concentration completely inhibited *E. coli* growth, while *E. coli* grew slowly in the presence of sub-MIC concentrations, eventually reaching the logarithmic and stationary phases. Previous research on AI-2 QS inhibition of *E. coli* has demonstrated that sub-MIC concentrations of berberine and matrine had no effect on *E. coli* growth whereas their MICs completely inhibit bacterial growth. Sub-MIC concentrations of these compounds were used to investigate QS-related phenotypic changes (Sun et al. 2019), which aligns with our study on the kinetic patterns of *E. coli* after exposure to various concentrations of PN. QS interference does not kill the bacteria but may influence biofilm formation or other QS-regulated phenotypes (Cady et al. 2012). Hence, concentrations below 1/2MIC were selected for anti-QS examination against *E. coli* strains.

EFFECT OF PN ON *E. coli* BIOFILM

The ability of *E. coli* strains to form biofilms poses significant challenges, particularly in the food industry, where biofilm formation contributes to food spoilage and disease transmission. The inhibitory efficacy of PN at 1/4 and 1/8MIC concentrations on *E. coli* biofilm formation, quantified by the crystal violet (CV) assay, is presented in Figure 5. The control groups (represented by dark blue bars) generally show higher biofilm formation compared to the PN-treated groups. Notably, *E. coli* 3.1 and EAEC PSU280 exhibited significant reductions in biofilm formation at both PN concentrations, suggesting that PN effectively inhibits biofilm formation in these strains. In contrast, strains such as *E. coli* 3.8, 5.5, and ATCC 25922 showed little to no change in biofilm formation under the same treatment conditions. Our previous study demonstrated that *E. coli* 3.1 and EAEC PSU 280 were classified as strong biofilm formers, while *E. coli* 3.8 and ATCC 25922, and *E. coli* 5.5 were classified as moderate and weak biofilm formers. In the current results, biofilm inhibition, as quantified by the CV assay, was observed primarily in the strong biofilm-forming strains. To further confirm the biofilm reduction, SEM analysis was conducted, and the results aligned with the findings of the CV assay. The SEM images, taken after 48 h of biofilm formation, show a strong anti-biofilm effect of PN at sub-MIC concentrations. The control groups showed thicker, multi-layered biofilm structures, while the treated groups exhibited a disrupted biofilm architecture with mostly single, separated cells. Importantly, the PN treatment did not affect the cell viability or morphology, as the bacterial cells retained their normal appearance despite the reduced biofilm formation (Figure 6). The differences observed between the CV assay and SEM results may arise from the limitations of the CV method, leading to potential inaccuracies. This is particularly evident in moderate and weak biofilm-forming strains such as *E. coli* 3.8, *E. coli* 5.5, and ATCC 25922, where the CV assay did not indicate significant biofilm reduction, while SEM images clearly showed a decrease in biofilm intensity. This variation suggests that while the CV assay is a useful tool for biofilm quantification, its limitations in detecting subtle structural changes underscore the need for complementary methods, such as SEM for a more accurate interpretation of biofilm inhibition. The SEM images also demonstrate that PN treatment reduced biofilm formation on glass surfaces, further confirming the anti-biofilm activity of PN. This finding supports previous research by Chaichana et al. (2023), which showed that the biofilm formation of *E. coli* strains was effectively inhibited by the *S. warneri* MG1_8-CFS with PN identified as a major active component.

Moreover, biofilm formation is a key virulence factor that allows *E. coli* to survive under stressful conditions by embedding cells in protective exopolysaccharide layers. This not only contributes to bacterial persistence but also enhances resistance to treatments, highlighting the importance of controlling biofilm formation (Galié et al. 2018; Olanbiwoninu & Popoola 2023). Over recent decades, natural anti-biofilm agents, such as plant extracts, essential oils, and bacterial metabolites, have gained attention for their efficacy against foodborne pathogens (Bazargani & Rohloff 2016; Blando et al. 2019; Gómez-Sequeda et al. 2020; Sandasi, Leonard & Viljoen 2010; Slobodníková et al. 2016). Mangrove environments are also remarkable sources for discovering new, safe, and eco-friendly natural bioactive agents, particularly from microorganisms, with potential applications across agriculture, pharmaceuticals,

industry, environment, and medical sciences (Liao et al. 2020). Sangkanu et al. (2017) reported that secondary metabolites (3-nitro-1,2-benzenedicarboxylic acid, hexadecanoic acid, quinoxaline-2-carboxamide, and pentadecanoic acid) produced by mangrove sediment-derived actinomycetes exert anti-biofilm activity against various antibiotic-resistant bacteria. As shown in this study, the sub-MIC concentration of PN derived from *S. warneri* MG1_8 disrupts mature biofilms of *E. coli*, offering promising potential for applications in preventing biofilm formation on solid surfaces across various industries.

INHIBITION OF SWIMMING MOTILITY

The impact of PN on the motility of *E. coli* strains was also evaluated, as motility plays a crucial role in *E. coli* biofilm formation. The motile ability of *E. coli* strains decreased after exposure to PN at sub-MIC concentrations in a concentration-dependent manner. At 1/4MIC of PN, swimming motility inhibition was significantly greater than at 1/8MIC (Figure 7). The swimming motility of *E. coli* strains 3.1, 3.8, 5.5, and ATCC 25922 was reduced by 75%, 78%, 56%, and 77% at 1/4MIC, respectively, and by 41%, 55%, 35%, and 64% at 1/8MIC, respectively.

QS plays a surprisingly large role in bacterial motility and other virulence factors which are critically pivotal to the food industry (Gopu, Meena & Shetty 2015). Motility allows *E. coli* to migrate and attach to new areas, facilitating biofilm expansion (Benyoussef et al. 2022). Our results indicated that PN effectively inhibited the swimming motility of all *E. coli* strains. Generally, natural compounds derived from plant-based extracts, such as grape seed, *Rosa rugosa* tea, Ginkgo biloba, oregano, and *Hb* bark, have been studied for their ability to inhibit bacterial motility and show potential to reduce *E. coli* motility (García-Heredia et al. 2016; Lee et al. 2014; Sheng et al. 2016). Furthermore, sub-MIC concentrations of polyphenols isolated from olive mill waste have been shown to significantly decrease both the swimming and swarming motility in *E. coli* K-12 (Carraro et al. 2014). In this study, natural PN synthesized by mangrove bacteria was able to inhibit the swimming motility of biofilm-forming *E. coli* strains suggesting that the anti-biofilm effect of PN on *E. coli* biofilms is closely related to its impact on motility.

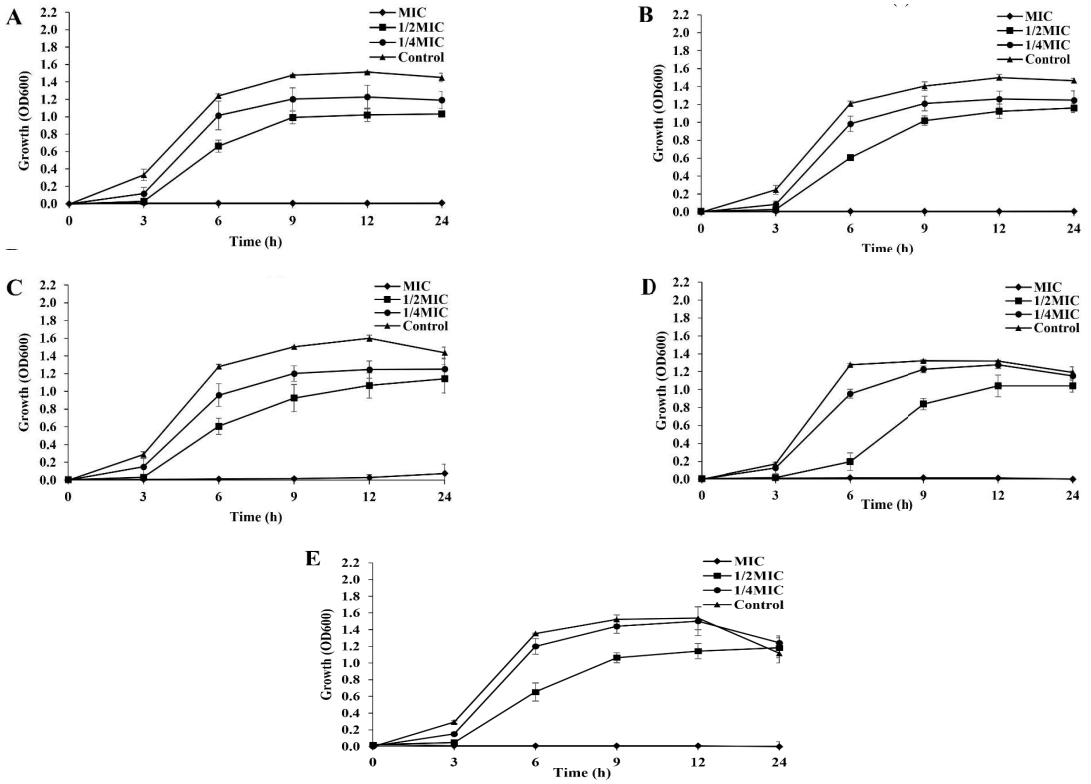

FIGURE 4. Growth curves of *E. coli* strains 3.1(A), 3.8 (B), 5.5 (C), ATCC 25922 (D), and EAEC PSU280 (E) treated with PN at different concentrations

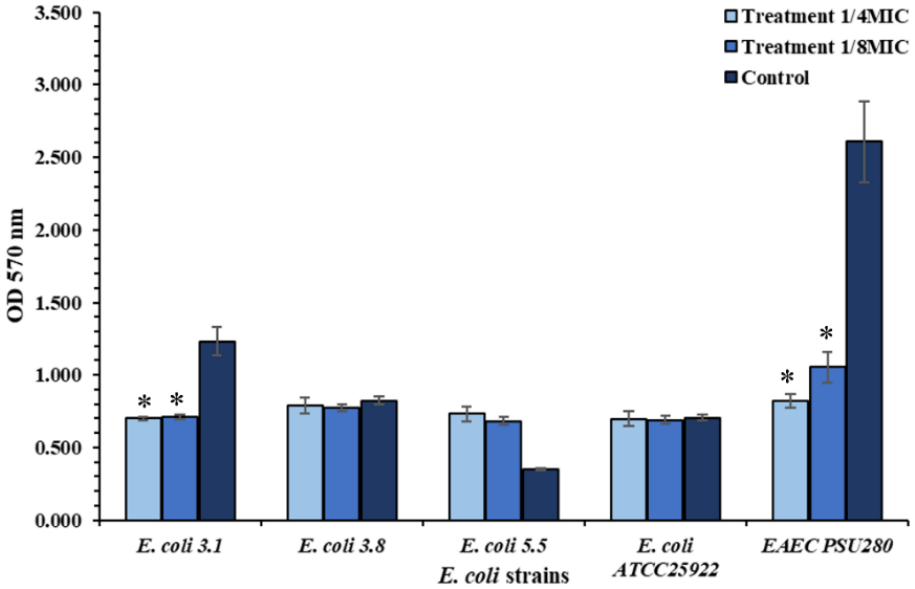

FIGURE 5. Biofilm formation of *E. coli* strains by sub-MIC concentrations (1/4MIC and 1/8MIC) of PN. Results are shown as the mean ± standard deviation of triplicate experiments. Significant differences (Dunnett, $P < 0.05$) compared to the control group

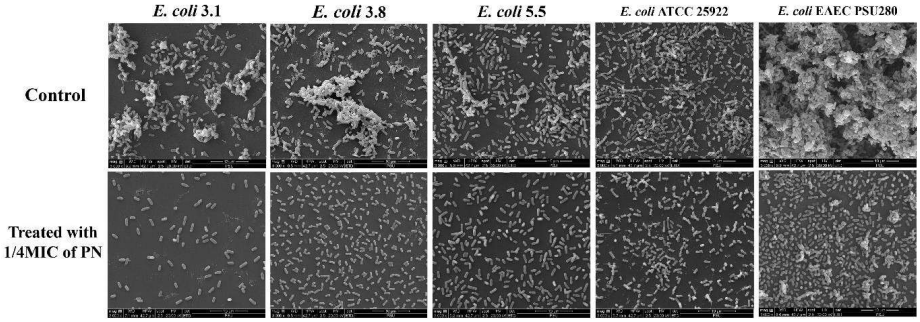

FIGURE 6. SEM images of *E. coli* biofilms in the absence (control groups) or presence (treatment groups) of PN, a major compound produced by *S. warneri* MG1_8, at 1/4MIC concentration

INHIBITORY EFFECT OF PN ON AI-2 ACTIVITY

In a previous report, we demonstrated that the *S. warneri* MG1_8-CFS crude extract at a 1/2MIC level inhibited the AI-2 activity of *E. coli* strains (Chaichana et al. 2023). PN, a major compound in the *S. warneri* MG1_8-CFS extract, was shown to inhibit AI-2 activity using the reporter strain *V. campbellii* ATCC BAA-1117. In this study, the inhibitory effects of 1/4MIC of PN on AI-2 activity were compared with control groups. As shown in Figure 8, 1/4MIC of PN significantly inhibited AI-2 activity of *E. coli* strains 3.1, 3.8, 5.5, ATCC 25922, and PSU 280 with reductions of 88%, 76%, 72%, 83%, and 79%, respectively. Similarly, the positive control (*V. campbellii* ATCC BAA-1119) represented a 63% reduction in AI-2 activity. The inhibitory activities of PN against AI-2 in *E. coli* strains ranged from 72% to 88% which were comparable to the 61% to 78% inhibition observed with the 1/2MIC of the *S. warneri* MG1_8-CFS crude extract. However, the inhibitory activity of 1/4MIC of PN was higher than the treatment with 1/2MIC of *S. warneri* MG1_8-CFS crude extract with no significant difference. The comparable effects on AI-2 activity reduction indicate the important role of PN as a major component produced by *S. warneri* MG1_8 with a high potential to be developed as an anti-QS agent.

The potential role of AI-2 QS in regulating bacterial persistence, growth, and virulence traits makes it an intriguing target for discovering novel biocontrol strategies to combat bacterial contamination (Coughlan et al. 2016;

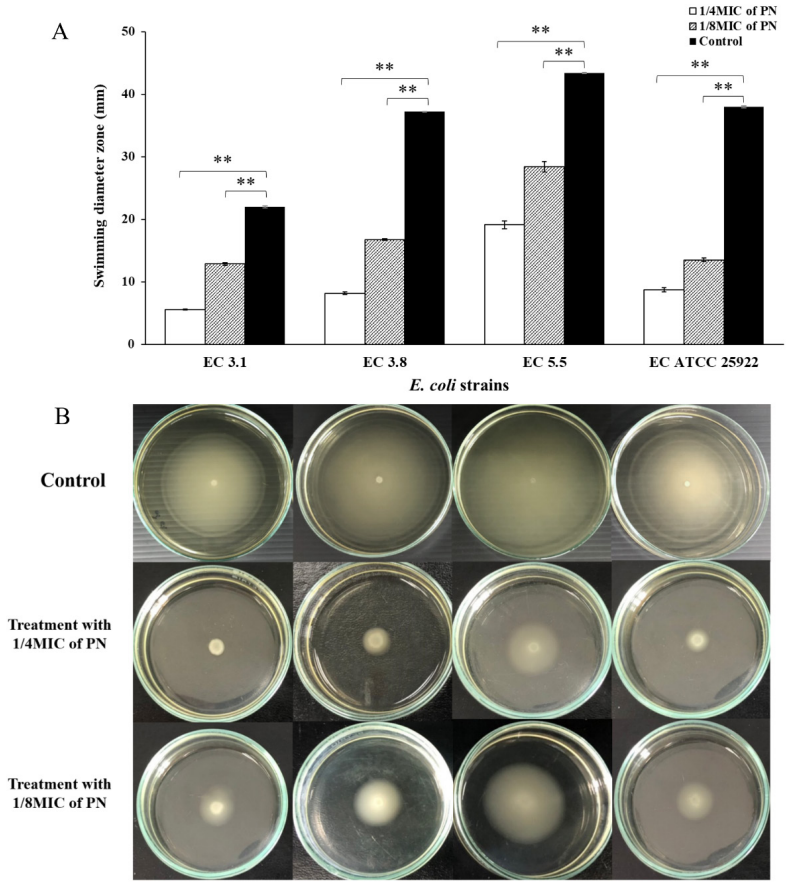

FIGURE 7. Swimming motility zone diameter (A) and images (B) of biofilm-forming *E. coli* strains in the presence of PN at sub-MIC concentrations and control. Significant differences (Dunnett, $P < 0.05$) compared to control

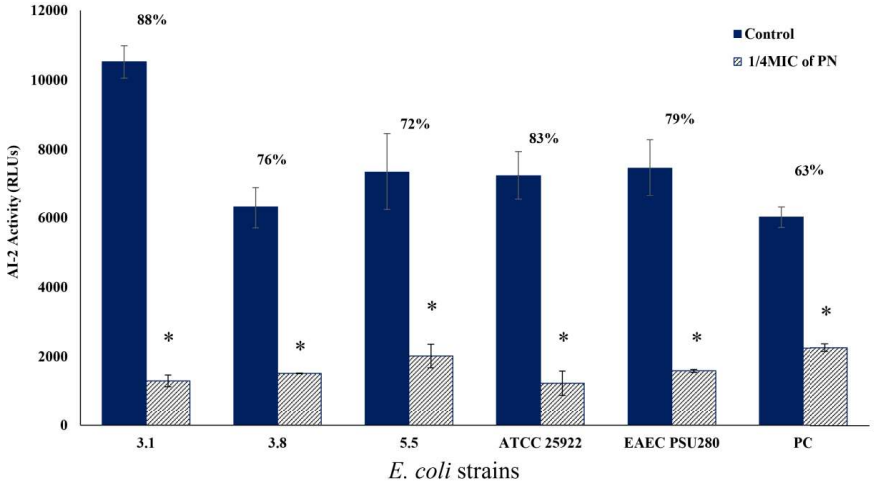

FIGURE 8. AI-2 signaling production and interference by PN at 1/4MIC concentration of biofilm-forming *E. coli* and *V. campbellii* ATCC BAA-1119 (Positive control). Significant differences (Dunnett, $P < 0.05$) compared to control

Zhu et al. 2022). In this study, we demonstrated the AI-2 reducing the effect of PN on biofilm-forming *E. coli* strains with the purpose of developing a candidate QS inhibitor agent. Until now, many attempts have been made to discover anti-AI-2 compounds from natural sources, especially among bacterial species. Probiotic bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp., have been studied for their ability not only to inhibit growth but also to interfere with QS in foodborne pathogens (Deng et al. 2022; Kim et al. 2012, 2008; Park et al. 2014). Therefore, our results suggest that PN has the potential to serve as an AI-2 inhibitor, preventing attachment and biofilm formation in *E. coli* strains which could help reduce bacterial contamination in foods and industrial productions.

PN derivatives are not only commercial interest but also medical interest for various applications. Previous studies have demonstrated that PN derivatives exhibit antibacterial, anti-QS, and anti-virulence activities against pathogens. Besides these, Heidari et al. (2017) found that pyridoxal lactohydrazone significantly inhibited virulence factors including motility, alginate and pyocyanin production, and susceptibility to H_2O_2 in *P. aeruginosa* suggesting that the pyridoxal ring has potential to inhibit the QS-related LasR protein. Moreover, the pyridoxal lactohydrazone showed anti-QS activity against the *Chromobacterium violaceum* CV026 biosensor strain.

QS has become a promising target for eradicating or reducing bacterial virulence which is a critical factor for bacteria to resist and persist in the food industry environment. Therefore, the results obtained from all experiments in this present study confirm that PN produced by mangrove bacteria *S. warneri* MG1_8 can act as a QSI agent to inhibit AI-2 QS-related traits, especially in preventing *E. coli* contamination by reducing biofilm formation, motility and AI-2 signaling in the food industry.

CONCLUSION

Current research has shown the anti-QS activities of *S. warneri* MG1_8-CFS extract against biofilm-forming *E. coli* strains. This mangrove bacterial isolate produces the anti-virulence substance which is identified as pyridoxine (PN). This compound at sub-MIC concentrations significantly inhibits biofilm formation, swimming motility, and AI-2 signaling production in *E. coli* strains 3.1, 3.8, 5.5, EAEC PSU 280, and ATCC 25922. These virulence factors are crucial for bacterial attachment and tolerance on surfaces in the food processing industry. Therefore, PN produced by *S. warneri* MG1_8 holds great potential as an alternative biocontrol strategy for managing *E. coli* contamination in the food industry.

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