Effects of Sulfide Flavors on AHL-Mediated Quorum Sensing and Biofilm Formation of *Hafnia alvei*

Hou Hongman, Wang Yifang, Zhang Gongliang, Zhu Yaolei, Xu Longquan, Hao Hongshun, Wang Yue, and Lu Meishan

Abstract: In this study, 10 different sulfide flavor compounds commonly used as food additives were screened for antiquorum-sensing activity. Among these, diallyl disulfide (DADS) and methyl 2-methyl-3-furyl disulfide (MMFDS) were found to exert the strongest inhibition against violacein production in *Chromobacterium violaceum* 026, the tested biosensor strain. DADS and MMFDS also inhibited the growth of *Hafnia alvei* H4, yielding MIC values of 48 and 41.6 mM, respectively. In addition, DADS and MMFDS also inhibited the ability of *H. alvei* H4 to produce acyl-homoserine lactone as demonstrated by the reduced level of C6-HSL in the supernatant of DADS-treated culture. At concentrations corresponding to 1/4 MIC, DADS, and MMFDS inhibited the swarming ability of *H. alvei* H4 by 73.50% and 76.43%, respectively, while having virtually no effect on cell growth. The same concentrations of DADS and MMFDS also completely inhibited the formation of biofilm. These antiquorum sensing effects of DADS and MMFDS involved changes in the expression of the quorum-sensing genes *luxI* and *luxR*. Quantitative RT-PCR analysis showed that the mRNA levels of both genes were significantly reduced by DADS and MMDFS at concentrations below their MICs. However, further test using a mutant strain of *H. alvei* lacking *luxR* (Δ*luxR*) revealed significant reduction in *luxI* mRNA level upon treatment of the strain with DADS or MMDFS, but no change in *luxR* mRNA level occurred when a *luxI*-lacking mutant (Δ*luxI*) was treated with these compounds. The result therefore suggested that the antiquorum-sensing effect of DADS and MMFDS against *H. alvei* H4 might operate mainly through the inhibition of *luxI* expression in the cells.

Keywords: acyl-homoserine lactone (AHL), biofilm formation, *Hafnia alvei*, quorum sensing, sulfide flavors

Practical Application: The sulfide flavors compounds used in this paper are commonly used in food processing in China and are listed in the national standard of Chinese food additives GB2760-2014. The application of sulfide flavors in food processing can enhance aroma and prevent food spoilage.

Introduction

Hafnia alvei, first identified by Moller in 1954, is a Gram-negative, rod-shaped, motile, flagellated, and facultative anaerobic bacillus (Bruhn et al., 2004; Møller, 1954). It is also a member of the acyl-homoserine lactone (AHL)-producing Enterobacteriaceae. *H. alvei* is an opportunistic pathogen and it is most commonly isolated from vacuum-packed dairy, meat, and fish products (Gram, Christensen, Ravn, Molin, & Givskov, 1999; Kagkli, Vancanneyt, Vandamme, Hill, & Cogan, 2007; Lindberg, Ljungh, Ahrné, & Löfdahl, 1998). In addition, several isolates of *H. alvei* from different food products are known to produce AHLs and form biofilm (Gamage, Luchansky, & Ingham, 1998; Janda & Abbott, 2006; Liu, Gray, & Griffiths, 2006). AHLs are generally fatty acid derivatives, and they form a major class of autoinducer-1 signals used by gram-negative bacteria to regulate diverse physiological traits, such as the production of virulence factors, biofilm formation, and swimming motility (Stevens, Schuster, & Rumbaugh, 2012). In the case of *H. alvei*, quorum sensing (QS) is essential for regulating virulence and other attributes, including biofilm formation and AHL production (Hou et al., 2017).

Some studies have indicated that *H. alvei* can produce N-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL), and N-butyryl-L-homoserine lactone (C4-HSL) (Hou et al., 2017; Tan, Yin, & Chan, 2014). AHL-mediated QS is widely prevalent among Gram-negative bacteria. This type of QS is composed of an AHL synthase (LuxI-type family protein) and an AHL reporter (LuxR-type family transcription regulator) (Kumar, Umesha, Prasad, & Niranjana, 2016). In bacteria, biofilm formation, AHL production and QS-regulated gene expression are complex processes, which among other processes, are regulated by the QS system (Ng & Bassler, 2009). Inhibitors of QS may be used to effectively prevent or eliminate biofilm formation, AHL secretion and QS-gene expression. DADS and MMFDS, both of which are thioether compounds, are food additives. They are strongly volatile materials that should be stored in a sealed preservation bottle. Recent research has shown that diallyl sulfides (diallyl monosulfide, dially disulfide, diallyl trisulfide, and diallyl tetrasulfide) possess inhibitory activity against QS (Rattanachaikunsopon & Phumkhachorn, 2008). Hence, it is plausible that quorum-sensing inhibitors might represent an antimicrobial strategy that could have significant

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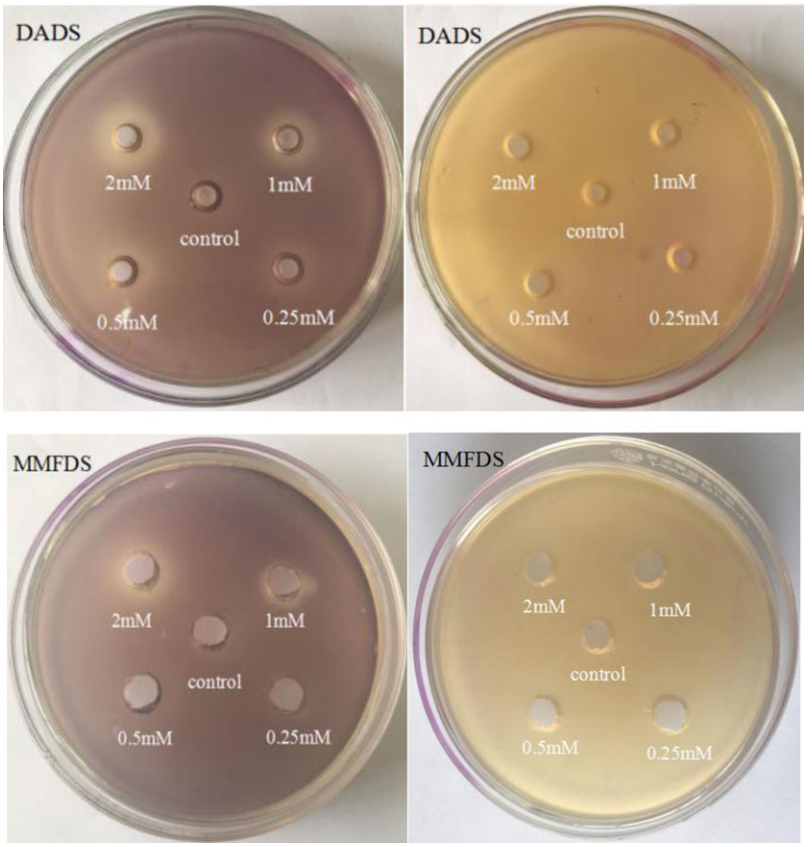


Figure 1—Effects of sulfide flavor compounds on violacein production of *Chromobacterium violaceum* 026
Production of violacein by *C. violaceum* 026 was monitored in the absence (control) or presence of different concentrations (0.25, 0.5, 1, and 2 mM) of DADS or MMFDS without and with C6-HSL. White zone around each well represents the absence of violacein.

impact on biofilm formation. Therefore, it seemed practical to search for efficient quorum-sensing inhibitors that can inhibit the spoilage of food products.

We have previously reported the extraction of 3 AHLs (C6-HSL, C4-HSL, 3-oxo-C8-HSL) from *H. alvei* H4 and their subsequent identification. C6-HSL was found to play a major role during the growth stage of the culture (Hou et al., 2017). In this study, we screened 10 sulfide flavors from different food sources for QS inhibitory activity using the *Chromobacterium violaceum* 026 screening system. We then analyzed the effects of these potential QS inhibitors on QS-regulated gene expression, biofilm and mortality of *H. alvei*.

Materials and Methods

Bacterial strains and culture conditions

H. alvei H4 was isolated from spoiled instant sea cucumber. The mutant strain lacking the luxI or luxR gene was constructed in our laboratory and stored at −80 °C in LB broth containing 28% glycerol. The bacteria were spread onto LB plates and incubated at 30 °C for 16 hr, and then maintained at 4 °C. *Chromobacterium violaceum* 026 (CV026) was used as the monitor strain to screen for QS inhibitors. It was provided by the Chinese Academy of Agricultural Sciences. CV026 is unable to synthesize its own C6-HSL but retains the ability to respond to exogenous C6-HSL and produce violacein (Stauff & Bassler, 2011). CV026 is usually used to detect the anti-QS activity of bioactive substances through their inhibition of AHL-mediated production of violacein. It is therefore an ideal strain for the screening of novel anti-QS compounds since the QS system consists of the *luxI/luxR* homologues *CviI/CviR* (McClean et al., 1997). C6-HSL was purchased from Sigma and used as an AHL standard. It has previously been shown to be the most significant QS signaling molecule detected during the first 12 hr of a *H. alvei* culture (Hou et al., 2017).

Screening for quorum-sensing inhibitors

For the screening of QS inhibitors, a simple protocol based on the inhibition of pigment production by *C. violaceum* or other bacterial strains was adopted (Kang, Han, Jeon, & Kim, 2016). *Chromobacterium violaceum* 026 was used as a biosensor strain to measure the level of AHL produced by *H. alvei*. It can produce the pigment violacein when exposed to exogenous AHL molecules. Aliquot (50 μL) of an overnight culture of CV026 was first added to 5 mL STA agar medium (0.65 g agar, 1.0 g Tryptone, 0.5 g NaCl, 100 mL deionized water). This followed by the addition of 20 μL C6-HSL standard (100 mg/mL) dissolved in dimethyl sulfoxide (DMSO). A control was also prepared which contained 20 μL DMSO without C6-HSL standard. The mixture was immediately poured into a sterile plate and allowed to solidify for 2 hr. After that, five wells (8 mm in diameter) were punched into the solidified agar, and each well was loaded with 50 μL of a sulfide flavor compound of different concentration (2, 1, 0.5, or 0.25 mM). A total of 10 different sulfide flavor compounds were used. The plates were then incubated at 30 °C for overnight.

Determination of minimum inhibitory concentration (MIC)

A stock solution of DADS or MMFDS was prepared by dissolving 10 mg of the compound in 1 mL DMSO. The solution was filtered through a 0.22-μm Millipore filter and stored at −20 °C. To determine the MIC values of DADS and MMFDS, the

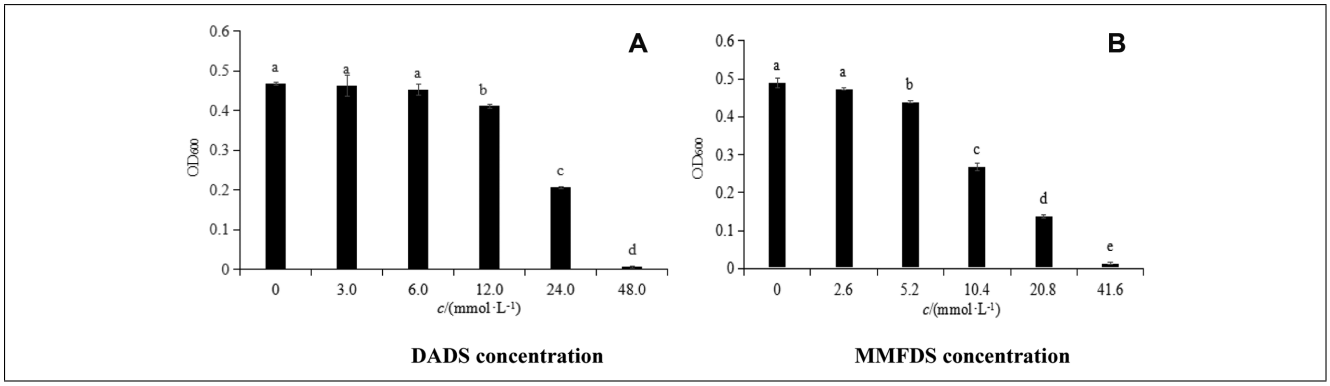


Figure 2–Effects of DADS and MMFDS on the growth of *H. alvei* H4.
***Hafina alvei* H4 was incubated with DAS (A) or MMFDS (B) for 24 hr and the growth rate was then measured by absorbance at A_{600}. Data are the means ± standard deviations from 3 experiments, each performed in triplicate.**

Table 1–Screening of different sulfide flavor compounds for anti-QSI activity.

Sulfide flavors	Anti-QS activity
DimethylTrisulfide (DMTS)	–
Methyl Propyl Disulfide (MPDS)	+
Methyl 2-methyl-3-furyl Disulfide (MMFDS)	++
Diallyl Disulfide (DADS)	+++
Ally Sulfide (DAS)	–
Dipropyl Disulfide (DPDS)	–
2-Methyl-3-(methylthio) furan (MMTF)	–
Methyl Furfuryl Disulfide (MFDS)	+
Furfuryl Isopropyl Sulfide (FFIS)	–
(2-methyl-3-furyl) Disulfide (2-MFDS)	–

No anti-QS activity detected (–); Low (+), moderate (++) and high (+++) levels of anti-QS activity.

stock solutions were serially diluted to yield working solutions of different concentrations (3, 6, 12, 24, and 48 mg/mL for DADS, and 2.6, 5.2, 10.4, 20.8, and 41.6 mg/mL for MMDFS) and 10 μL of each working solution was added to 1 mL LB medium. This was followed by the addition of 10 μL of an overnight *H. alvei* H4 culture (OD_{600}nm = 0.4) and 24 hr of incubation at 30 °C. The cell density of *H. alvei* H4 in each culture was monitored by measuring the OD_{600} at every 4-hr interval (Zhu, Huang, Zhang, Feng, & Li, 2015). The MIC value was determined as the lowest concentration of the compound that inhibited growth.

AHL extraction and analysis by HPLC

Different concentrations of DADS and MMFDS corresponding to 1/4, 1/8, 1/16, and 1/32 of their MICs were prepared and 100 μL of each was added to 99 mL LB medium followed by the addition of 1 mL of an overnight culture of *H. alvei* H4. The samples were cultured at 30 °C for 12 hr. A blank control was also prepared which contained 100 μL DMSO without DADS or MMFDS. After incubation, the samples were centrifuged at 8000 × *g* and 4 °C for 15 min, and the supernatant from each sample was thoroughly mixed with an equal volume of ethyl acetate containing 0.1% acetic acid (v/v) followed by incubation at 25 °C for 2 hr with shaking at 180 rpm. After that, the ethyl acetate layer was removed and freeze-dried under vacuum, and the residue was dissolved in 1 mL ultra-pure water and further dried in a vacuum drier (Ravn, Christensen, Molin, Giveskov, & Gram, 2001). The residual AHL extract was dissolved in 100 μL of 25/75 (v/v) acetonitrile/water and subjected HPLC analysis as described later.

AHL in the *H. alvei* H4 extract was detected by HPLC according to a previously described method (Chong et al., 2012). An aliquot (10 μL) of the crude AHL extract was injected into a reverse phase C18 column (Luna II C18 column; particle size, 10 μm; 250 × 10 mm) connected to a ShimadzuUFLP Prominence HPLC system that was coupled to a photodiode array detector set at 250 nm. The column was eluted with 50% methanol for 30 min at a flow rate of 5 mL/min. The elution profile was compared with that of the C6-HSL standard (10 mg/mL) obtained under the same conditions. The presence of C6-HSL in the AHL extract was identified according to the retention time of the peak corresponding to the peak of the C6-HSL standard. The area of the peak was calculated to determine the amount of the compound.

Effects of QSIs on AHL production in *H. alvei* H4

For the qualitative and quantitative detections of AHL in the *H. alvei* H4 extract, a similar experiment was carried out as for the AHL extraction described above, except that after the mixing of DADS or MMDFS with the *H. alvei* H4 cultures, the cultures were incubated for 4, 8, or 12 hr, and AHL was extracted from these cultures and used as a source of AHL for the subsequent assay. In the assay, 40 mL of an overnight CV026 culture was mixed with 160 mL melted LB agar medium (0.8% agar), and the resulting mixture was poured into 10 sterile plates and left to solidify. Ten-millimeter wells were then punched in the agar plate, and each well was loaded with 50 μL of the prepared AHL extract. The plates were incubated at 30 °C for overnight and the extent of purple pigment production was then assessed visually.

Swimming motility assay

Swimming motility was performed according to a previously described method (Rashid & Kornberg, 2000). In brief, a swimming agar plate consisting of 1% tryptone, 0.5% NaCl, and 0.3% agarose supplemented with DADS or MMFDS (final concentrations corresponded to 1/32, 1/16, 1/8, and 1/4 MIC) was prepared and 3 μL of an overnight culture of *H. alvei* H4 (OD_{600} = 0.5) was placed at the center of the plate. For control, the plate contained the same medium but without DADS or MMFDS. All the plates were incubated at 30 °C for 48 hr and the migration distances of the cells were recorded by measuring the diameters of the colony zones.

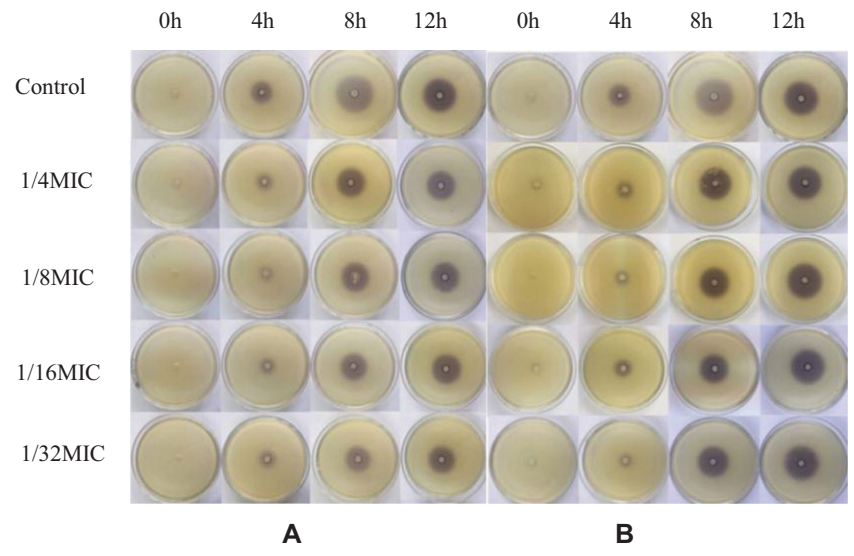


Figure 3–Effect of DADS and MMFDS on AHL production of *H. alvei* H4
Production of violacein by CV026 in the presence of *H. alvei* extract only (control) or plus various sub MICs of DADS (A) or MMDFS (B).

Table 2–HPLC analysis of AHL in the extract prepared from *H. alvei* H4 grown in the presence of DADS or MMFDS.

sample	C6 standard	*H. alvei* H4	DADS-1/4MIC	DADS-1/2MIC	DADS-MIC	MMFDS-1/4MIC	MMFDS-1/2MIC	MMFDS-MIC
Retention time (min)	19.387	19.326	19.399	19.390	–	19.395	19.388	–
Concentration (μM)	1000	400	201	87	–	156	61	–

Table 3–Effect of DADS and MMDFS on the swarming motility of *H. alvei* H4.

QSI	Concentration	Migration zone (mm)	Inhibition (%)	OD600nm
None	0	68.3 ± 0.1	0	1.6 ± 0.1
DADS	1/32MIC	49.8 ± 0.2	27.09	1.6 ± 0.1
	1/16MIC	37.5 ± 0.1	45.14	1.6 ± 0.1
	1/8MIC	24.1 ± 0.1	64.76	1.6 ± 0.1
	1/4MIC	18.1 ± 0.1	73.50	1.6 ± 0.1
MMFDS	1/32MIC	52.3 ± 0.2	23.47	1.6 ± 0.1
	1/16MIC	35.0 ± 0.6	48.80	1.6 ± 0.1
	1/8MIC	21.2 ± 0.4	68.91	1.6 ± 0.1
	1/4MIC	16.1 ± 0.2	76.43	1.6 ± 0.1

Biofilm formation assay

The quantification of biofilm formation was performed using a regular 96-well microtiter plate as described previously (Truchado et al., 2012), but with minor modifications. Briefly, an overnight culture of *H. alvei* H4 was inoculated into LB medium using an inoculum to medium ratio of 1:100. This culture was then added to a 96-well plate (Corning Inc., Corning, NY, U.S.A.) using 200 μL per well. This was followed by the addition of DADS or MMFDS (final concentrations of 3, 6, 12, and 24 mM). For negative control, DMSO was added to a final concentration of 1% instead of DADS or MMFDS. The plate was incubated at 30 °C for 24 hr and the absorbance of the plate was then measured at 600 nm to determine the extent of cell growth. The biofilm produced was visualized after the plate was stained with crystal violet solution (0.1%). The percent of biofilm formation inhibited by DADS or MMFDS was calculated according to the following equation:

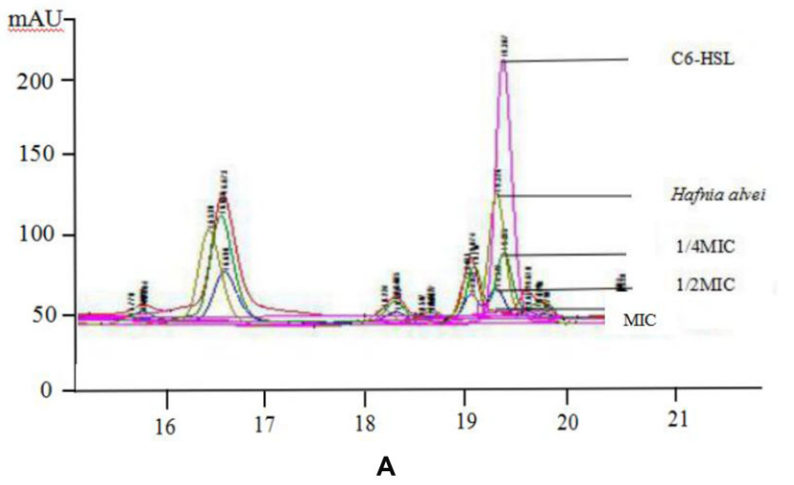
$$1 - (OD_{590}\ \text{oftest}/OD_{590}\ \text{of untreated control}) \times 100\%.$$

Biofilm analysis by scanning electron microscopy (SEM)

For scanning electron microscopy (SEM), *H. alvei* H4 was incubated in LB medium for overnight and then adjusted to a final density of about 1.0×10^7 CFU/mL. First, 0.1 mL of the *H. alvei* H4 culture was dispensed onto a sterile plastic coverslip placed inside a 6-well microtiter plate (Corning Inc., Costar). This was followed by the addition of 1 mL fresh LB medium and 10 μL DADS or MMFDS (concentration corresponding to 1/4 or 1/2 MIC) at 30 °C for 60 hr. After that, the biofilm deposited on the coverslip was analyzed by SEM. The coverslip was prepared for SEM analysis using a previously described method (Nithya, Begum, & Pandian, 2010). In brief, the coverslip was gently washed with sterile distilled water, and the biofilm was fixed for 2 hr in a solution containing 2.5% glutaraldehyde followed by washing with 0.1 M sterile phosphate-buffered saline. Finally, the coverslip was subjected to dehydration under a graded series of tert-butanol. This was followed by critical point drying, gold sputtering and SEM examination (Quanta 450, Waltham, MA, U.S.A.) performed at 3.0 kV and 10000× magnification.

RNA isolation and cDNA synthesis

Wild-type *H. alvei* H4 and two mutant strains, Δ*R* (lacking *luxR* gene) and Δ*luxI* (lacking *luxI* gene), were cultured in LB medium supplemented with DADS or MMFDS for 12 hr. Prior to RNA isolation, the cells were treated with RNA protect reagent (Qiagen, Valencia, CA, U.S.A.) as recommended by the



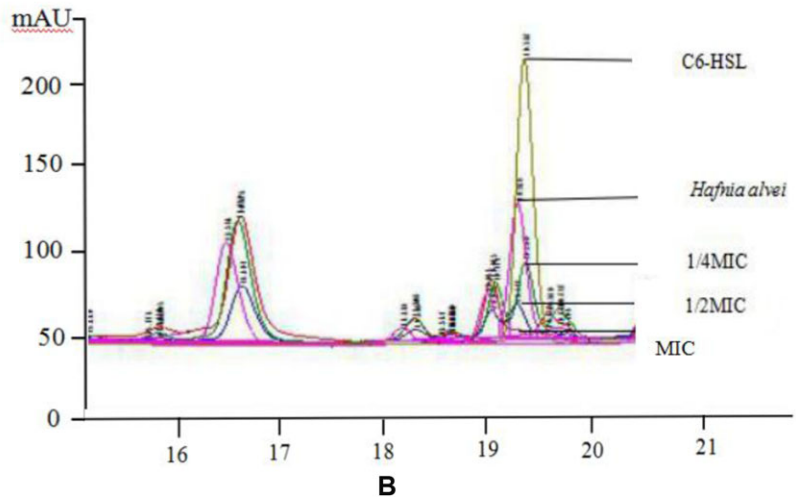


Figure 4–HPLC analysis of the effects of QSIs on the production of C6-HSL in *Hafnia alvei*
(A) DADS; (B) MMFDS.

manufacturer. Total RNA was extracted from the cell pellet using TRI Reagent (Sigma) according to the manufacturer's instruction, and then treated with a TURBO DNA-free Kit (Life Technologies, Carlsbad, CA, U.S.A.) to remove residual DNA. Before synthesizing the complementary DNA (cDNA) strands, RNA concentration in the sample was quantified with a NanoDrop ND-1000 spectrophotometer (Implen, Munich,Germany). First-strand cDNA was synthesized from 2 μg of total RNA using a High Capacity RNA-to-cDNA Kit (Life Technologies) according to the manufacturer's instruction (Myszk, Schmidt, Olejnik-Schmidt, Leja, & Czaczyk, 2014).

Quantitative real-time PCR

The expression of *luxI* and *luxR* genes in the planktonic culture was detected by SYBR® Green JumpStart™ TaqReadyMix™. The thermal cycling program used was as follows: denaturation at 95 °C for 2 min; 40 cycles consisting of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C. The housekeeping gene 16 SrRNA was used as an internal reference for data normalization. Threshold values, which were used for threshold cycle (Ct) determination, were generated automatically by the Bio-Rad software. The comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) was used to analyze relative mRNA level.

Statistical analysis

All data were expressed as means ± standard deviations. Analysis of variance (ANOVA) was conducted, and differences between variables were tested for significance by one-way ANOVA with the Tukey test by using SPSS Version 16.0 (SPSS Inc., IBM, Armonk, NY, U.S.A). Statistical significance was considered at the $P < 0.05$ level.

Result

Screening sulfide flavor compounds for quorum-sensing inhibitors

Chromobacterium violaceum is often used as an indicator organism for the indirect screening of QS inhibitors. Violacein, the purple pigment produced by CV026 is regulated by the CviIR-dependent quorum-sensing system and stimulated in the presence of QS signaling molecule such as C6-HSL. Therefore, interfering with this signaling will lead to the inhibition of violaceum production, and this is considered direct evidence of QS interference (Fawcett et al., 1997). Preliminary screening of 10 sulfide flavor compounds for anti-QS activity was performed in liquid culture using CV026 as the biosensor (Table 1). The extent of anti-QS activity exerted by each compound was determined by the diameter of the inhibitory zone around the point of application (>20 mm =

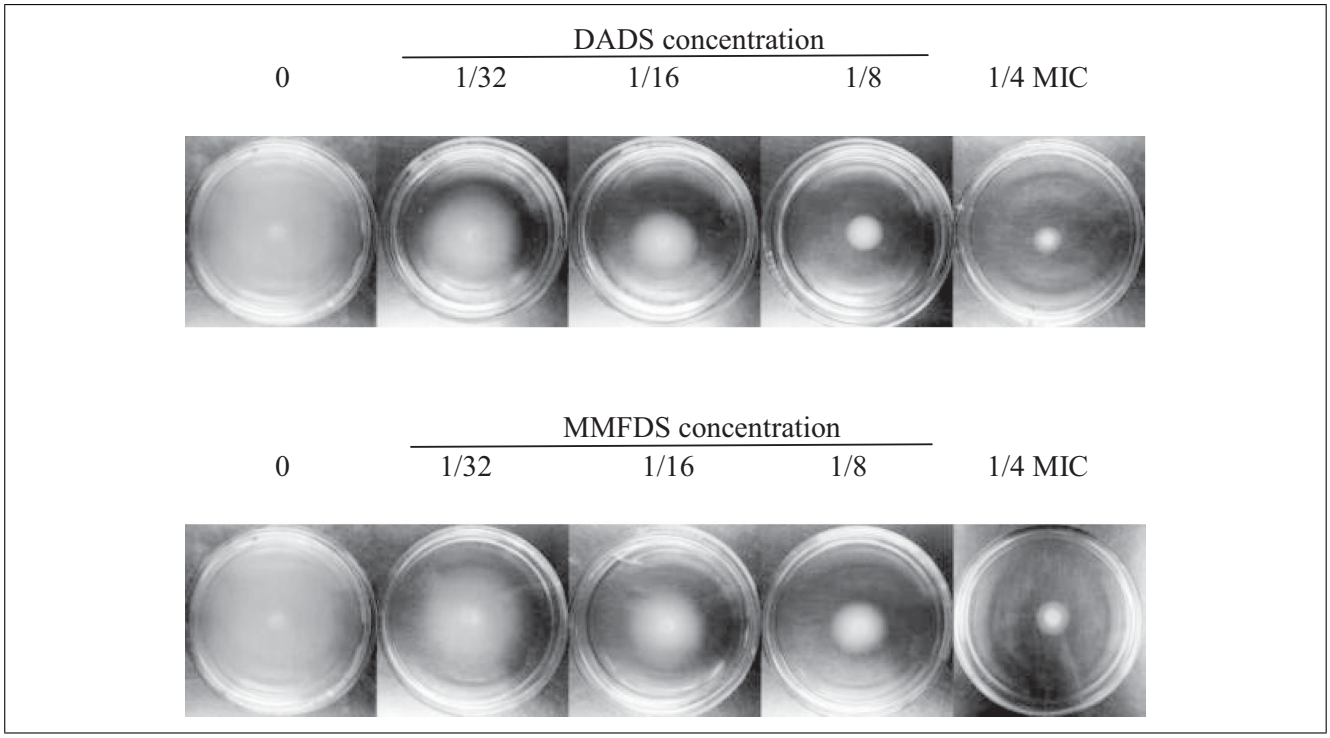


Figure 5–Inhibition of *H. alvei* swarming motility by DADS and MMFDS.
***H. alvei* motility was determined in soft agar plate containing either no QSI (DMSO only) or various concentrations of DADFS or MMDFS as indicated.**

high; 15 to 20 mm = moderate; and <15 mm = low). Among the 10 compounds tested, DADS and MMFDS caused much more reduction in violacein production than the rest (Table 1). The inhibition of violacein production by DADs and MMFDS was further tested in agar-plate culture using various concentrations (0.25 to 2 mM) of the compounds. A dose-dependent inhibition of violacein production was obtained, with higher concentrations of the compounds resulting in greater inhibition (Figure 1). In contrast, no inhibition was observed in the absence of DADS or MMDFS. These results indicated that both DADS and MMDFS acted as QS inhibitors by interfering with the C6-HSL-mediated QS signaling pathway, consequently leading to greatly reduced production of violacein. A similar inhibitory effect on violacein production in *Chromobacterium cyminum* has been reported previously, but the inhibitory agent used was clove oil (Khan, Zahin, Hasan, Husain, & Ahmad, 2010).

Effect of QSIs on the growth of *H. alvei* H4

To further examine the QS inhibitory activity of DADS and MMFDS, both compounds were tested for their effect on the growth of *H. alvei* H4. The growth of *H. alvei* was unaffected by DADS at 3.0 to 12.0 mM or by MMFDS at 2.6 to 10.4 mM (Figure 2). The minimum inhibition concentration (MIC) values of DADS and MMFDS were determined to be 48 mM and 41.6 mM, respectively. Based on the MIC values, all subsequent experiments were conducted at the sub-MICs of DADS and MMFDS.

Effect of QSIs on AHL production in *H. alvei* H4

To determine the effects of DADS and MMFDS on the production of AHLs in *H. alvei*, the violacein assay was performed using LB-agar plates. *Hafnia alvei* H4 incubated with DADS for 4, 8, and 12 hr exhibited reduced production of violacein compared to *H. alvei* incubated without DADS or MMDFS (control) (Figure 3A). Compared to the control, *H. alvei* H4 incubated with MMFDS for 4 hr exhibited obvious reduction in violacein production, but longer incubation resulted in no obvious change in violacein production (Figure 3B). The results of these experiments were consistent with those obtained with C6-HSL (Figure 1), further demonstrating the anti-QS activity of both DADS and MMFDS. According to the results of a previous study, the concentration of C6-HSL is highest among the various quorum-sensing signaling molecules detected in *H. alvei* H4 culture during the first 12 hr of incubation (Hou et al., 2017). Thus, in order to study the effect of DADS and MMFDS on the production of AHL, the biosensor experiment, which only provided a qualitative analysis, was followed by HPLC analysis to identify the specific QS signaling molecule and quantify the change in its level caused by DADS and MMFDS. The biosensor experiment only showed the effect of DADS and MMFDS on the production of all AHLs. It could not distinguish which specific AHL molecules were responsible for inducing the production of violacein in CV026 (Figure 3). The AHLs extracted from the supernatant of a 12-hr *H. alvei* H4 culture were analyzed by HPLC. Various peaks were resolved and the major peak had a retention time of 19.3 min, corresponding to the retention time of C6-HSL standard. When the culture was treated with DADS or MMDFS, the intensity of this peak decreased with increasing concentrations of the compound (Figure 4). However, this peak was virtually absent when the concentration of DADS or MMDFS used corresponded to its MIC (Table 2). Thus C6-HSL production was obviously inhibited by increasing the concentrations of DADS or MMFDS in the culture. Inhibition of AHL production in the presence of DADS and MMFDS may be due to the suppression of *luxR* expression caused by *luxI*, either at the transcription or

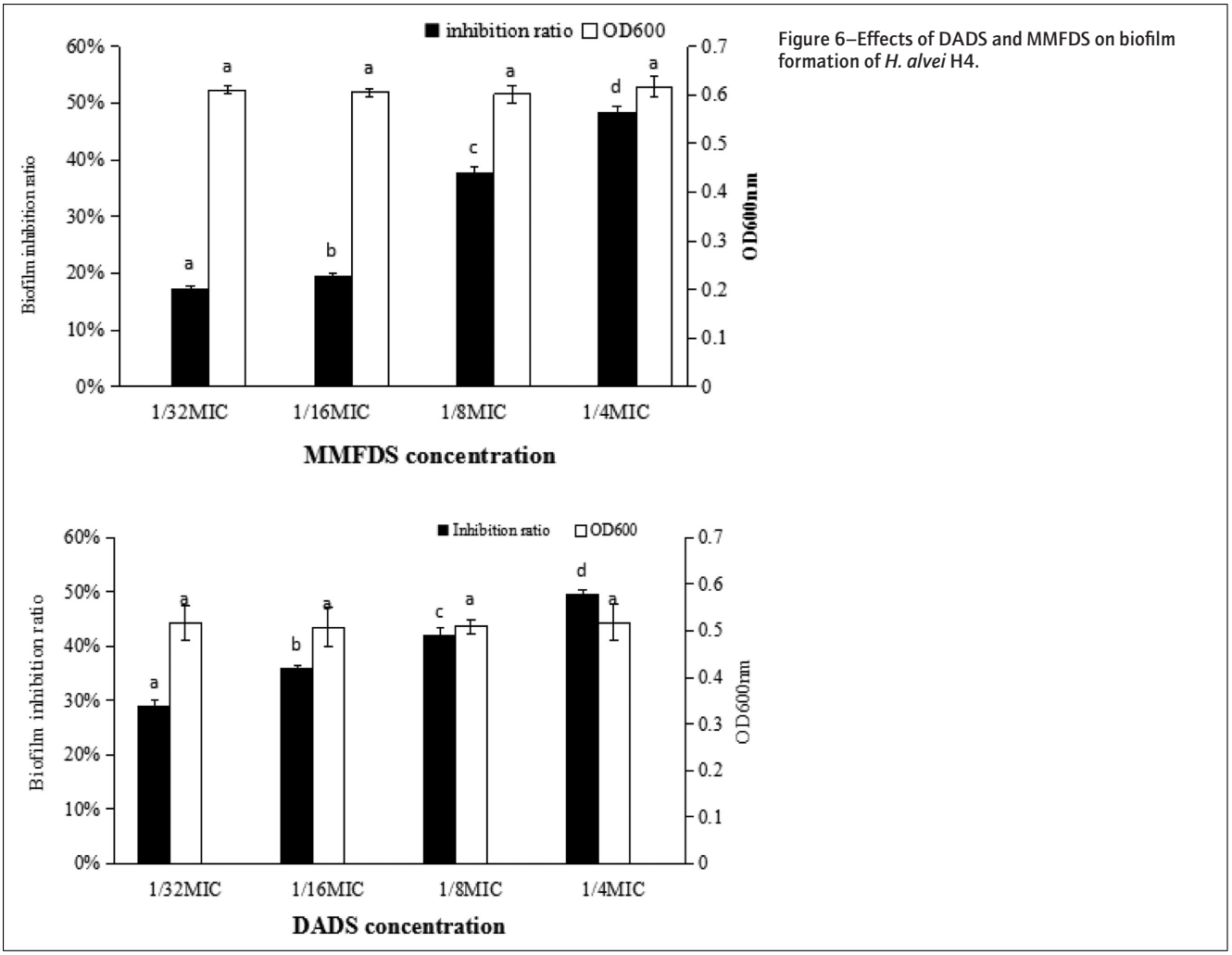


Figure 6–Effects of DADS and MMFDS on biofilm formation of *H. alvei* H4.

translation level. Previous study has been demonstrated that both AHL and *LasR* are essential for achieving maximal activation of the *lasB* gene (Asfahl & Schuster, 2017).

Effect of QSIs on swarming motility

Flagellar-dependent swimming motility is related to biofilm formation, which is coordinately regulated by AHL-mediated QS (Morris, Finney, Rajachar, & Kohn, 2004). To investigate whether the motility of *H. alvei* H4 might be involved in biofilm formation, swarming motility was analyzed by agar diffusion assay using medium containing different concentrations of DADS or MMFDS. In the presence of DADS or MMFDS, the motility of *H. alvei* H4 was significantly inhibited as revealed by the reduced migration distance from the origin of inoculation (Figure 5), indicating that the inhibition was dependent on the concentration of the compound. After 24 hr of treatment with DADS at a concentration corresponding to one-fourth of the MIC, the motility of *H. alvei* H4 was reduced by 73.5% (Table 3). Similarly, the motility of *H. alvei* H4 was reduced by 76.4% after treatment with MMFDS at a concentration corresponding to one-fourth of the MIC. Despite the decrease in migration distance, the optical density of *H. alvei* H4 remained unaffected by DADS and MMFDS, suggesting that DADS and MMFDS inhibited the motility of *H. alvei* H4 without affecting its growth. A previous study looking at the effect of mango leaf extract on the swarming motility of *P. aeruginosa* also reported a dose-dependent decrease in motility with respect to the concentration of mango extract (Husain et al., 2017). Swarming motility has been reported to contribute to the early stages of *P. aeruginosa* biofilm formation, which plays an essential role in surface colonization (Shrout et al., 2006). Therefore, inhibiting the motility of *H. alvei* could indeed disrupt its ability to form biofilm.

Effect of QSIs on biofilm formation

Considering that biofilm formation is partially controlled by the QS system, a crystal violet assay was performed in a 96-well plate to explore the effects of DADS and MMFDS on *H. alvei* H4 biofilm formation. After incubation with DADS or MMDFS at 1/32 to 1/4 the MIC for 24 hr, the formation of biofilm was reduced by 18% to 48% in the case of DADS treatment, and 29% to 50% in the case of MMDFS treatment (Figure 6B). On the other hand, no effect on bacterial growth was observed. In many Gram-negative bacteria, cell motility and biofilm formation are regulated by AHL-mediated QS (Davies et al., 1998; Givskov et al., 1996; Manefield et al., 1999; Nadell, Xavier, Levin, & Foster, 2008). At sub-inhibitory concentration, eugenol, a synthetic food flavouring agent, can reduce the biofilm formation of *Staphylococcus aureus* by 17% to 86% and inhibit the metabolic activity of the cells (Nasser, Fohad, Iqbal, & Mohammad, 2017). Other sulfide flavor compounds that are used as food additives have been shown

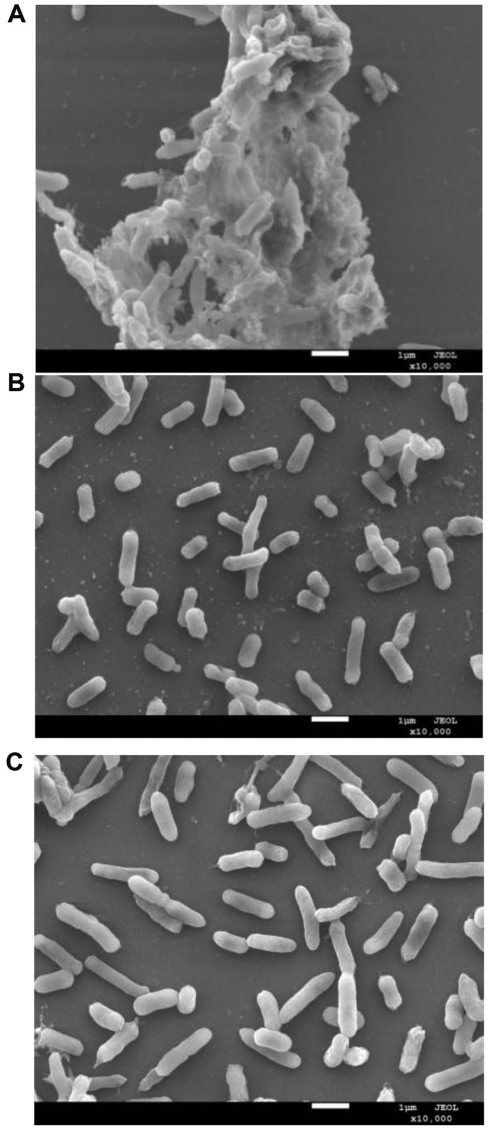


Figure 7–SEM images showing the biofilm formed by *H. alvei* H4 in the absence and presence of QS inhibitors.
(A) *H. alvei* (control); (B) DADS at 1/4 MIC; (C) MMDFS at 1/4 MIC.

to inhibit the swarming motility and biofilm formation of *E. coli, L. monocytogenes, P. aeruginosa*, and *S. aureus* (Borges et al., 2014). Isothiocyanates, 2-phenylethy lisothiocyanate (PEITC) and allylisothiocyanate (AITC) are the most commonly used sulfide flavors.

The structure of *H. alvei* H4 biofilm was examined by SEM. Untreated *H. alvei* H4 (control) formed a densely packed biofilm consisting of layers of exopolysaccharide, and the bacterial cells were embedded within these layers of exopolysaccharide (Figure 7A). No biofilm was formed by *H. alvei* H4 treated with DADS or MMFDS at 1/4 the MIC (Figure 7B and C). After DADS or MMFDS was added to the culture, the ability of *H. alvei* H4 to adhere to plastic coverslips was significantly reduced because the cells secreted less exopolysaccharides. Such interference with biofilm formation might be due to reduced surface adhesion or other subsequent steps in the biofilm formation process. There are 3 stages in the process of biofilm formation: adhesion, evolution and maturation (Ryu & Beuchat, 2005). Our data appeared to suggest that DADS and MMFDS might inhibit the process of biofilm formation at the adhesion stage, probably through interfering with AHL-mediated QS signaling. Previous study has reported that biofilm formation by *H. alvei* can be inhibited by furanone, and that *H. alvei* 071 AHL-lacking mutant cannot form biofilm (Viana, Campos, Ponce, Mantovani, & Vanetti, 2009). The effect of AHL is thus necessary for biofilm formation. Another compound that has also been shown to reduce biofilm formation is coumarin, which is effective at reducing the extent of biofilm formed by *E. tarda* and *V. anguillarum*, both of which possess AHL-QS homologous genes (Milton et al., 1997; Morohoshi, India, Kato, Kanai, & Ikeda, 2004).

Effect of quorum-sensing inhibitors on *LuxI/R g*ene expression

Fluorescence real-time quantitative PCR was performed to investigate the effects of DADS and MMFDS on the expression of the QS genes, *luxI* and *luxR*. The expression levels of *luxI* and *luxR* were calculated relative to their levels during the planktonic growth mode (the first 12 hr of growth). *Hafnia alvei* cells treated with DADS or MMFDS exhibited reduced levels of *luxR* and *luxI* transcripts compared to nontreated cells (controls), and the reduction was significant for all concentrations (1/4, 1/8, 1/16, and 1/32 MICs) of DADS and MMFDS tested (Figure 8A and B). This indicated that both QS inhibitors could negatively regulate the expression of *luxI* and *luxR* genes. In the case of *H. alvei* H4 treated with 1/16 MIC and 1/8 MIC of DADS, the rise in *luxR* transcript levels above the level obtained for *H. alvei* treated with 1/32 MIC of DADS was rather unexpected. However, these levels were still much lower compared to the level of the control cells. To eliminate the possibility that *luxI* and *luxR* might influence each other's expression, the expression of the two genes in the presence of either quorum-sensing inhibitor was also evaluated using a mutant strain of *H. alvei* lacking either the *luxI* gene (Δ*luxR*) or *luxR* gene (Δ*luxI*). The result showed that DADS and MMFDS significantly inhibited the expression of *luxI* and in a concentration-dependent manner, as shown by the reduced level of *luxI* transcript in the Δ*luxR* mutant treated with DADS or MMFDS (Figure 8E and F). However, no change in the level of *luxR* mRNA occurred when Δ*luxI* was treated with DAS or MMFDS compared to the untreated Δ*luxI*, suggesting that these two QS inhibitors might not affect the expression of the *luxR* gene (data not shown). Overall, the inhibitory effect exerted by DADS and MMFDS on the QS system appeared to be accomplished through the inhibition of *luxI* expression. This indicated that DADS and MMFDS might preferentially target the *luxI* gene product. Tannic acid is a common synthetic food additive as are DADS and MMFDS. Study based on bacterial luminescence and AHL production has suggested that tannic acid directly interferes with the transcription of the QS genes *lasI* and *rhlI,* and potentially affects the expression and functions of the Lux proteins (Chang et al., 2014).

Conclusion

Two potential QS inhibitors, DADS and MMFDS, were identified through screening a number of sulfide flavor compounds produced by *H. alvei* H4. Both DADS and MMFDS inhibited QS by suppressing AHL secretion, swarming motility, and biofilm formation, and this might be linked to their ability to inhibit the expression of the QS gene *LuxI*. DADS and MMFDS might therefore be considered as potential QS inhibitors with a broad spectrum of activity. However, the precise mechanism by which

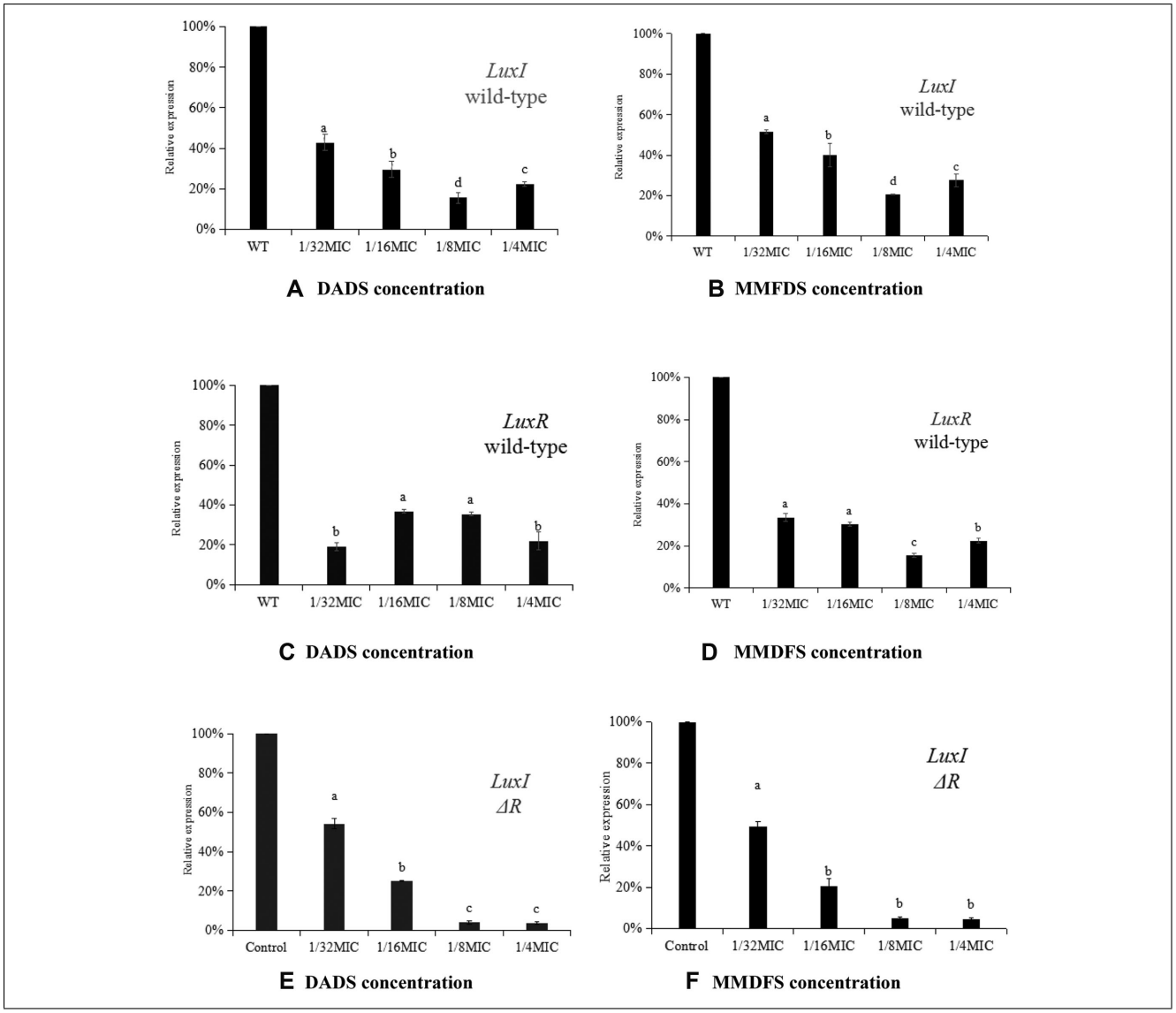


Figure 8–Effects of DADS and MMDFS on the expression of *luxI* and *luxR genes* in *H. alvei* H4. *LuxI and luxR* mRNA levels of *H. alvei* H4 were measured by *q*RT-PCR following treatment without or with DADS or MMDFS at the indicated concentrations.

DADS and MMFDS exert their inhibitory effect on QS requires further study.

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Author Contributions

Hou H.M. conceived the project and provided all constructs to generate the research. Wang Y.F. carried out the experiments and prepared and analyzed all the data and figures of the paper, and Hou H.M. and Wang Y.F. wrote the paper. Zhang G.L. and Zhu Y.L. provided the gene knock-out strains. Xu L.Q. and Lu M.S. carried out SEM analysis of biofilm formation, Hao H.S. and Wang Yue trained Wang Y.F. in swarming analyze.

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