Research article

Indole-3-acetic acid increases the survival of brine shrimp challenged with vibrios belonging to the Harveyi clade

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ABSTRACT

Vibrios belonging to the Harveyi clade (including closely related species such as *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus*) are important pathogens of aquatic organisms. In this study, we investigated the use of indole-3-acetic acid to control disease caused by Harveyi clade vibrios. Indole-3-acetic acid, which can be produced by various seaweeds and microalgae, was added to the rearing water of brine shrimp larvae challenged with twelve different Harveyi clade *Vibrio* strains. Indole-3-acetic acid significantly decreased the virulence of ten of the strains without any effect on their growth. The latter is important as it will minimize the selective pressure for resistance development. The survival rate of brine shrimp larvae increased with 1.32-fold to ~~5.2~~4.8-fold upon treatment with 400 µM indole-3-acetic acid. Additionally, indole-3-acetic acid significantly decreased the swimming motility in ten of the strains and biofilm formation in eight of the strains. The mRNA levels of the *pirA* and *pirB* toxin genes were decreased to 46% and 42% by indole-3-acetic acid in the AHPND-causing strain *V. parahaemolyticus* M0904. Hence, our data demonstrate that indole-3-acetic acid has the potential to be an effective virulence inhibitor to control infections in aquaculture.

Key words: motility; biofilm; acute hepatopancreatic necrosis disease (AHPND); quorum sensing; indole signaling; IAA

1. INTRODUCTION

According to FAO (2022), world fisheries and aquaculture production is estimated to have reached about 177.8 million tonnes in 2020, of which 87.5 million tonnes (49%) came from aquaculture. Shrimp farming is an important sector of the aquaculture industry. However, vibriosis, a disease caused by various vibrios, results in massive mortality of cultured shrimp (Novriadi 2016). Vibrios belonging to the Harveyi clade (containing the closely related species *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus*) lead to significant losses in shrimp aquaculture, causing up to 100% mortality (Austin and Zhang 2006; Defoirdt et al. 2007; Karunasagar et al. 1994; Prayitno and Latchford 1995). Recently, acute hepatopancreatic necrosis disease (AHPND) has originated and spread in Asian and South-American countries (Tran et al. 2013; Soto-Rodriguez et al. 2015), and led to a global loss of more than USD 40 billion in the shrimp industry (Kumar et al. 2021). AHPND was shown to be caused by specific strains of *V. parahaemolyticus* and *V. campbellii* containing a plasmid encoding homologs of the *Photorhabdus* insect-related (Pir) toxins PirA and PirB (Dong et al. 2017b; Lee et al. 2015a).

In order to deal with bacterial diseases, the conventional approach is to use antibiotics and disinfectants (Scott et al. 2019). The frequent use of these compounds has resulted in the development and spread of resistance in pathogenic bacteria, and antibiotics commonly used in aquaculture are no longer effective in some cases (Defoirdt et al. 2011). For example, a *V. harveyi* train, the cause of mass mortality of *Penaeus monodon* larvae, was resistant to cotrimoxazole, chloramphenicol, erythromycin and streptomycin (Karunasagar et al. 1994). Further, AHPND-causing *V. parahaemolyticus* strains from Mexico were found to carry the *tetB* gene encoding tetracycline resistance, and a *V. campbellii* strain from China was reported to contain several antibiotic resistance genes (Dong et al. 2017a; Kumar et al. 2021). Consequently, we need novel strategies replacing antibiotics in order to control bacterial diseases in aquaculture (Defoirdt et al. 2011). Antivirulence therapy, i.e. disarming pathogens

by inhibiting the production of virulence factors (i.e. molecules that enable the pathogens to infect the host) rather than killing them, is a new therapeutic strategy that currently is being explored (Clatworthy et al. 2007; Defoirdt et al. 2013). Indole signaling is a potential target for antivirulence therapy because it decreases the production of virulence factors in various bacterial pathogens (Lee et al. 2015b). It has been documented that indole decreased virulence-related phenotypes in *Vibrio anguillarum*, *Vibrio crassostreae*, *Vibrio tasmaniensis*, *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus* (Li et al. 2014; Yang et al. 2017; Zhang et al. 2022a; Zhang et al. 2022b). In addition, indole improved the survival of host animals (mussel larvae, fish larvae and shrimp larvae) challenged with these pathogens (Li et al. 2014; Zhang et al. 2022a; Zhang et al. 2022b). However, indole appeared to be toxic to brine shrimp larvae and mussel larvae at the concentration that led to optimal inhibition of virulence-related phenotypes (200 μM) (Yang et al. 2017).

Indole-3-acetic acid, the most biologically active auxin plant hormone, is produced by plants (Lehmann et al. 2010; Ljung 2013), bacteria (Stirk et al. 2013), seaweeds (Zhang et al. 1993) and microalgae (Mazur et al. 2001). The compound has an important role in plant-pathogen interactions, particularly in the bacterial infection progress, as some phytopathogens can promote infection by manipulating auxin homeostasis in plants (Ludwig-Müller 2015; Navarro et al. 2006; Spoel and Dong 2008). Recently, indole-3-acetic acid was reported to decrease biofilm formation and exopolysaccharide production in *V. campbellii* BB120, and pretreatment of the pathogen with indole-3-acetic acid increased the survival of brine shrimp larvae challenged with the pathogen (Yang et al. 2017). Furthermore, indole-3-acetic acid decreased the mortality of brine shrimp larvae challenged with a *V. harveyi* strain when combined with undecanoic acid (Salini et al. 2019). However, at this moment, it is not clear to what extent there is a variability in different strains with respect to the response to indole-3-acetic acid. In this study, we aimed at further exploring the impact of indole-3-acetic acid on the virulence of

different vibrios belonging to the Harveyi clade by testing the impact of indole-3-acetic acid on the survival of brine shrimp larvae challenged with 12 different strains belonging to the Harveyi clade. Further, the impact of indole-3-acetic acid on bacterial growth, swimming motility and biofilm formation were also investigated.

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture conditions and chemicals

The strains that were used in this study are shown in **Table 1**. The strains were grown in Luria-Bertani medium containing 35 g/L of sodium chloride (LB_{35}) at 28°C with shaking (120 min^{-1}). Cell densities were measured with a spectrophotometer at 600 nm. Indole-3-acetic acid, (Sigma-Aldrich, Belgium), was dissolved in water at 100, 200, 400, 500, 600, 800 and 1000 mM. For all experiments, each treatment received the same volume of water (i.e. 1 µL/mL).

2.2. Determination of the impact of indole-3-acetic acid on bacterial growth

To determine the effect of indole-3-acetic acid on the growth of the vibrios, overnight grown cultures were inoculated into fresh LB_{35} medium at an OD_{600} of 0.01. Indole-3-acetic acid was added in different concentrations (0, 100, 200, 500 and 1000 µM, respectively). Then, 200 µl volumes of these diluted cultures were transferred to the wells of a polystyrene 96-well plate and incubated for 24h at 28℃. The OD_{600} of each culture was measured every hour with a Tecan Infinite M200Pro instrument. The growth curves were measured for three replicate cultures.

2.3. Hatching of axenic brine shrimp larvae

Five hundred milligrams of *Artemia franciscana* cysts (EGVR Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 45 ml sterile tap water for 1 h. Sterile cysts and larvae were obtained by decapsulation as described by Marques et al. (2005). In brief, 25 ml of NaOCl (50%) and 1.65 ml of NaOH (32%) were added to the suspension of hydrated cysts to facilitate decapsulation. The decapsulation was stopped after 2 min by adding 35 ml of $Na_2S_2O_3$ (10 g/L). Filtered aeration (0.22 µm) was provided during the whole reaction.

The decapsulated cysts were washed with sterile artificial seawater containing 35 g/L of instant ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France) and re-suspended in a bottle containing 1 L of sterile artificial seawater. The cysts were incubated for at least 28 h at 28°C with aeration and constant illumination (2000 lux). The sterility of the cysts was verified by inoculating 1 ml of rearing water into 9 ml of LB_{35} and checking for turbidity after incubating at 28°C for 24 h. After 28 h of incubation, groups of 30 larvae were transferred into sterile 50 ml tubes containing 30 ml of sterile artificial seawater. Finally, the tubes were put on a rotor (4 rotations per min) and kept at 28°C. All manipulations were done in a laminar flow hood in order to maintain sterility.

2.4. Brine shrimp challenge tests

The impact of indole-3-acetic acid on the virulence of vibrios was assessed in a standardized challenge test with gnotobiotic brine shrimp larvae (Defoirdt et al., 2005). Firstly, we explored the effects of different concentrations of indole-3-acetic acid on the virulence of the model pathogen *V. campbellii* BB120. Briefly, *V. campbellii* BB120 and indole-3-acetic acid were added to the brine shrimp rearing water at the start of the experiment. The inoculum concentration of *V. campbellii* BB120 was 10^6 CFU/mL, and indole-3-acetic acid was added at

200, 400, 600, 800 and 1000 µM, respectively. At the start of the challenge test, a suspension of autoclaved LVS3 bacteria (Verschuere et al., 1999) in sterile artificial seawater was added to all of the cultures at 10^7 cells/mL as feed. Brine shrimp cultures to which only water and feed were added, were used as controls. The survival of the larvae was determined after 2 days of incubation.

In the second and third brine shrimp challenge test, vibrios and indole-3-acetic acid were added to the rearing water at the start of the experiment. The concentrations of indole-3-acetic acid were 200 and 400 µM, respectively. The inoculum concentrations of vibrios were 10^6 CFU/mL for *V. parahaemolyticus* M0904, CAIM170, *V. campbellii* S01, LMG21361, LMG21362, LMG22889 and LMG22890; and 10^7 CFU/ mL for *V. harveyi* VIB571 and *V. campbellii* S01 (because these strains did not cause significant mortality when added at 10^6 CFU/mL). Autoclaved LVS3 bacteria (10^7 cells/mL) were added as feed to all cultures at the start of the challenge test. Brine shrimp cultures to which only water and feed were added, were used as controls. The survival of the larvae was determined after 2 days of incubation.

For all challenge tests, each treatment was performed in triplicate. The sterility of the control treatments was each time checked at the end of the test by inoculating 1 mL of rearing water of the control treatment into 9 mL of LB_{35} and checking for turbidity after incubating at 28°C for 2 days. The concentrations of the vibrios in the brine shrimp rearing water were determined at the end of the challenge tests by plate counting on LB_{35} agar.

2.5. Determination of swimming motility

Swimming motility was determined on LB_{35} soft agar plates containing 0.2% agar (Yang and Defoirdt, 2015). After autoclaving, the LB_{35} soft agar was cooled down to approximately 50°C, after which 200 µM of indole-3-acetic acid was added. The same volume of water was added

to the control agar. Subsequently, the agar was poured into petri plates, after which the plates were air-dried with open lid for 15 min. The *Vibrio* strains were cultured overnight in LB_{35} broth and diluted to an OD_{600} of 1. Five µL aliquots of these suspensions were applied to the center of the soft agar plates (six replicates per treatment). The plates were incubated upright at 28 ℃ and the motility zones were measured after two days.

2.6. Determination of biofilm levels

Biofilm levels were quantified with crystal violet staining as described previously (Stepanović et al., 2007). Briefly, overnight grown cultures of the strains were diluted to an OD_{600} of 0.1 in LB_{35} medium without or with indole-3-acetic acid (at 200 and 400 µM). Two hundred microlitre volumes of these suspensions were transferred into the wells of a polystyrene 96 well plate, after which the plates were incubated at 28℃ for 24h without agitation. After the incubation, unattached cells were removed by washing with PBS for three times. The remaining attached cells were fixed with 200 µl methanol per well. The methanol was removed after 20 min, after which the plates were air-dried. Subsequently, biofilms were stained with 200 µl per well of a crystal violet solution (1%) for 15 min. The wells were then rinsed with water until the washings were free of the stain, after which the plates were air-dried. Subsequently, bound crystal violet was dissolved in 200 µl of 95% ethanol per well. After 30 min, absorbance at 570 nm was measured with a Tecan Infinate M200Pro instrument. Sterile medium was used as negative control, and the reported values are blank-corrected.

2.7. RNA extraction and quantitative reverse transcriptase PCR (RT-qPCR)

To detect the effect of indole on the expression of the AHPND toxins *pirA* and *pirB*, overnight cultured *V. parahaemolytius* M0904 and *V. campbellii* S01 were diluted (1% v/v) in fresh LB_{35} medium without or with indole-3-acetic acid at 400 µM. Each culture was grown for 6 h at 28℃ in triplicate (Zhang et al., 2022a).

RNA extraction and reverse transcriptase real-time PCR were performed as described previously (Yang and Defoirdt, 2015). RNA was extracted with the SV Total RNA Isolation System (Promega), after which DNA contamination was removed with DNase (Thermo Scientific Rapid Out DNA Removal Kit). The RNA quantity was measured with a spectrophotometer (NanoDrop Technologies) and adjusted to 200 ng/µl. RNA quality was checked with agarose gel electrophoresis. The RNA samples were stored at -80℃.

cDNA was synthesized with the ProtoScript (r) II First Strand cDNA Synthesis Kit (New England biolabs) according to the manufacturer's instructions. Briefly, 1 µg RNA and 2 µl random primer were mixed with Nuclease-free H_2O to a total volume of 8 µl. The samples were denatured at 65℃ for 5 min and immediately put on ice. After this, 2 µl of 10 × Enzyme Mix and 10 µl of 2 × Reaction Mix were added. The cDNA synthesis reactions (20 µl volumes) were incubated at 25℃ for 5 min, followed by 42℃ for 60 min and 80℃ for 5 minutes.The cDNA samples were cooled down to 4℃. Finally, they were checked with PCR and stored at -20℃ until further use.

Real-time qPCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, Gent, Belgium) with the Luna Universal qPCR Master Mix (New England biolabs). The primers that were used are shown in **Table 2**. For each treatment, three biological replicates were analysed. The *recA* gene was used as a control for *V. parahaemolyticus* M0904, and the *rpoA* gene was used as a control for *V. campbellii* S01. The efficiencies of the PCR for *pirA*

and *pirB* were similar to those of *rpoA* and *recA*. Consequently, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA levels of *pirA* and *pirB*.

2.8. Statistics

Data were analysed using the SPSS statistical software (version 27). Independent samples t-tests were used to compare treatments receiving indole-3-acetic acid to the corresponding treatments without indole-3-acetic acid. P values are indicated in the Figures and Tables.

3. RESULTS

3.1. Determination of the impact of indole-3-acetic acid on the growth of the *Vibrio* strains

In a first experiment, we determined the impact of indole-3-acetic acid on the growth of the strains in LB_{35} medium. Indole-3-acetic acid did not affect growth of any of the strains for concentrations up to 1000 µM (**Fig. S1**). This is an important point as we aim to use indole-3-acetic acid for antivirulence therapy, which aims at blocking the virulence of pathogens without affecting their viability (in order to minimize the selective pressure for resistance development).

3.2. Determination of the impact of indole-3-acetic acid on the virulence of the strains towards gnotobiotic brine shrimp larvae

In the first test, we determined the best dosage of indole-3-acetic acid. Yang et al (2017) have shown that pretreatment with 200 µM indole-3-acetic acid resulted in a higher protective effect

on brine shrimp larvae against *V. campbellii* BB120 than 50 and 100 µM. Based on this result, we decided to use indole-3-acetic acid at concentrations of 200, 400, 600, 800 and 1000 µM to test the effect on the virulence of the model pathogen *V. campbellii* BB120. The results indicated that the survival of challenged brine shrimp larvae was proportional to the concentration of indole-3-acetic acid, with a significantly increased survival at 400 to 1000 µM (**Fig. 1A**). The density of *V. campbellii* BB120 in the brine shrimp rearing water after two days of challenge was not affected by 200 and 400 µM indole-3-acetic acid, but was decreased in the treatments with higher concentrations of indole-3-acetic acid (**Fig. 1B**).

In the second and third test, 200 and 400 µM of indole-3-acetic acid were added into the brine shrimp rearing water and the larvae were challenged with the 12 different strains. The results demonstrated that 200 µM indole-3-acetic acid significantly increased the survival of brine shrimp larvae for five of the strains (~~*V. parahaemolyticus* M0904, *V. harveyi* VIB571, and *V. campbellii* LMG21361, LMG2889 and LMG22890~~). For 5 other strains (CAIM170, E022, S01, LMG 21362 and LMG 22888), we also recorded an increased survival in the presence of 200 µM indole-3-acetic acid, but the differences were not significant due to high variabilities. Further, 400 µM indole-3-acetic acid significantly increased the survival of challenged brine shrimp larvae for 10 of the strains (~~the 5 strains mentioned above and *V. harveyi* VIB645 and LMG22893, and *V. campbellii* S01, LMG21362 and LMG22888~~ M0904, VIB571, VIB645, LMG 22893, S01, LMG 21361, LMG 21632, LMG 22888, LMG 22889 and LMG 22890), resulting in between 1.2-fold (for strain CAIM170) and 4.8-fold (for strain LMG 21362) higher survival (**Table 3**). Importantly, indole-3-acetic acid had no impact on bacterial densities in the brine shrimp rearing water at these concentrations (**Fig. 2**).

3.3. Determination of the impact of indole-3-acetic acid on swimming motility and biofilm levels of the *Vibrio* strains

We further investigated the impact of indole-3-acetic acid on those virulence factors that have been shown before to be affected by indole and by indole-3-acetic acid in *V. campbellii* strain BB120 (Yang et al. 2017). Firstly, we tested the impact of indole-3-acetic acid at 200 µM on swimming motility of the strains. The results demonstrated that indole-3-acetic acid significantly decreased the swimming motility in ten of the strains (i.e. M0904, CAIM170, VIB571, E022, LMG22893, S01, LMG21362, LMG22888, LMG22889 and LMG22890).

In the second *in vitro* experiment, the impact of 200 and 400 µM of indole-3-acetic acid on biofilm levels produced by the 12 Harveyi clade strains was investigated. Indole-3-acetic acid at 200 µM was found to significantly decrease the biofilm formation for 5 of the strains (VIB571, VIB645, S01, LMG21362, LMG 22890), whereas 400 µM indole-3-acetic acid significantly reduced biofilm formation for 8 of the strains (including the 5 strains mentioned above and CAIM170, E022 and LMG22893) (**Fig. 4**).

3.4. Determination of the impact of indole-3-acetic acid on mRNA levels of the *pirA and pirB* toxin genes

To further explore how indole-3-acetic acid affects the production of virulence factors of the AHPND-causing strains, relative mRNA levels of the *pirA* and *pirB* toxin genes were determined. After culturing in the presence of 400 µM indole-3-acetic acid for 6 h, the mRNA levels of *pirA* and *pirB* were decreased to 46% and 42%, respectively, in *V. parahaemolyticus* M0904, while there were no differences for *V. campbellii* S01 (**Fig. 5**).

4. DISCUSSION

It has been reported before that pretreatment of *V. campbellii* BB120 with indole-3-acetic acid significantly increased the survival of challenged brine shrimp larvae (Yang et al. 2017). In that study, a pretreatment of the pathogen was used in order to ensure that any increased survival that would be observed was due to inhibition of the virulence of the pathogen and not due to any direct effect of indole-3-acetic acid on the brine shrimp larvae. To further investigate the use of indole-3-acetic acid as a virulence inhibitor to protect shrimp larvae from vibrios belonging to the Harveyi clade, in this study, a standardized challenge test was performed (Defoirdt et al. 2006), in which indole-3-acetic acid was added directly to the rearing water of brine shrimp larvae. The results showed that indole-3-acetic acid at 400 to 1000 µM significantly improved the survival of brine shrimp larvae challenged with *V. campbellii* BB120, while there was a small (and non-significant) effect at 200 µM. The effect of 200 µM indole-3-acetic acid was smaller than what we observed before when pretreating *V. campbellii* BB120 with indole-3-acetic acid. This might indicate that indole-3-acetic acid needs to be added as a preventive treatment. The result obtained with 200 µM indole-3-acetic acid is similar to what Salini et al. (2019) reported, as they also found that indole-3-acetic acid at 30 µg/ml (about 171 µM) only had a small effect on brine shrimp larvae challenged with a *V. harveyi* strain. Meanwhile, indole-3-acetic acid did not affect the growth of *V. campbellii* BB120 in LB_{35} medium for concentrations up to 1000 µM. However, at concentrations of 600, 800 and 1000 µM, it decreased the cell density in the gnotobiotic brine shrimp larval rearing water after two days of challenge. This difference indicated that the bacteria are more sensitive to inhibition by indole-3-acetic acid when they are in a more harsh environment (with lower levels of nutrients) such as the brine shrimp cultures. Given the idea of antivirulence therapy (disarming pathogens without affecting their viability), concentrations of 200 and 400 µM indole-3-acetic acid were selected to do further research. The results indicated that indole-3-acetic acid improved the

survival of brine shrimp larvae challenged with most of the strains without any effect on their cell density, suggesting that the protection offered by indole-3-acetic acid was not due to growth inhibition of the pathogens.

We previously reported that indole signaling, a bacterial cell-to-cell communication mechanism, controls the virulence of different vibrios (Li et al. 2014; Yang et al. 2017; Zhang et al. 2022a; Zhang et al. 2022b). In this study, we investigated the use of indole-3-acetic acid as an analogue of indole in order to protect shrimp from vibriosis, using brine shrimp as a model. We found that indole-3-acetic acid has a similar effect as indole, i.e. improving the survival of brine shrimp larvae challenged with different Harveyi clade vibrios (Yang et al. 2017; Zhang et al. 2022a). Importantly, indole-3-acetic acid showed less toxicity to brine shrimp larvae than indole (which is toxic at 200μM), and the brine shrimp larvae survived well even at 1000 μM of indole-3-acetic acid (**Fig. 1A**). This suggests that the use of indole-3-acetic acid is safe for the animals. Furthermore, because indole-3-acetic acid can be produced by various microalgae (Lu and Xu 2015; Stirk et al. 2013), this virulence inhibitory activity might (partly) explain the beneficial effect of greenwater, i.e. water containing high levels of microalgae. Indeed, empirical evidence indicates that there is a lower occurence of disease in green water aquaculture systems (De Schryver et al. 2014; Natrah et al. 2014). Our results suggest that the production of indole-3-acetic acid might be (partly) responsible for this beneficial effect, although further research is needed in order to determine what levels of indole-3-acetic acid can be obtained in the digestive tract of aquatic animals upon consumption of microalgae.

During an infection, production of virulence factors plays an important role as it enables the pathogens to infect and damage the host. Since indole-3-acetic acid showed a protective effect on brine shrimp larvae against Harveyi clade vibrios, the production of virulence factors was further explored, focussing on swimming motility, biofilm formation, and the expression of the toxin genes *pirA* and *pirB*. Biofilm formation is a mechanism that is involved in serious

bacterial infections (Joo and Otto 2012); it enables bacteria to withstand the host response, and to be less susceptible to antibiotics and disinfectants (Jacques et al. 2010). For instance, the persistence and survival in shrimp hatcheries of *Vibrio harveyi* were attributed to the ability of the bacterium to form biofilms with resistance to disinfectants and antibiotics (Karunasagar et al. 1994). Given the elevated rate of resistance to antibiotics in biofilms, there has been more effort towards the discovery and characterization of novel natural anti-biofilm agents, such as plant extracts, microbes from the marine environment, and cyanobacteria and microalgal compounds (Arunkumar et al. 2020). In our previous study, we found that indole inhibited biofilm formation of *V. campbellii*, *V. harveyi*, *V. parahaemolyticus*, and *V. crassostreae* (Zhang et al. 2022a; Zhang et al. 2022b), which is consistent with the impact of indole-3-acetic acid in this paper, which significantly reduced biofilm formation of eight of the strains of Harveyi clade vibrios.

Numerous pathogenic bacteria have developed a large array of motility mechanisms to detect available resources, move towards favorable environments and avoid detrimental conditions (Josenhans and Suerbaum 2002; Wadhwa and Berg 2022). The swimming motility inhibitor phenamil has been reported before to significantly decrease the virulence of *V. campbellii* towards gnotobiotic brine shrimp larvae (Yang and Defoirdt 2015). Furthermore, indole has also been shown to decrease the swimming motility in *V. campbellii* (Yang et al. 2017), *V. harveyi*, and *V. parahaemolyticus* (Zhang et al. 2022a). Indole has been shown to decrease motility in *V. campbellii* by decreasing the mRNA levels of the lateral flagellar genes *lafA* and *lafK* (Yang et al., 2017). Similar to the effect of indole, indole-3-acetic acid decreased swimming motility in 11 Harveyi clade *Vibrio* strains in this study. It needs to be confirmed, however, whether indole-3-acetic acid decreases motility via the same mechanism as indole.

AHPND is a severe shrimp disease that is caused by strains carrying a virulence plasmid that encodes the *pirA* and *pirB* genes (Lee et al. 2015a). In addition to *V. parahaemolyticus* strains,

several strains belonging to other *Vibrio* species, including *V. campbellii*, *V. owensii*, and *V. harveyi* were also reported to cause AHPND (Dong et al. 2017a; Kondo et al. 2015; Liu et al. 2015). We previously reported that indole decreased the mRNA levels of the *pirA* and *pirB* toxin genes in the AHPND-causing strains *V. parahaemolyticus* M0904 and *V. campbellii* S01 (Zhang et al. 2022a). In this study, indole-3-acetic acid significantly decreased the mRNA levels of *pirA* and *pirB* in *V. parahaemolyticus* M0904 but did not affect *V. campbellii* S01. The two different results indicate that the mechanisms by which indole-3-acetic acid affects the virulence is different in different strains. The result for strain S01 is in contrast to what we observed for indole, which decreased the levels of *pirA* and *pirB* mRNA in this strain under the same conditions (Zhang et al. 2022a). In further research, it might be interesting to test the impact of indole-3-acetic acid on mRNA levels of the *pirA* and *pirB* genes in a time series as the effect of indole-3-acetic acid might be dependent on the growth pahse of the bacteria. Hence, it might be possible that indole-3-acetic acid affects pirA and pirB mRNA levels in strain S01 when when sampled in another growth phase. It would also be interesting to determine the effect of indole-3-acetic acid on PirA and PirB protein levels as in the end, it is the proteins that are causing the disease symptoms.

In conclusion, we found that indole-3-acetic acid significantly improved the survival of brine shrimp larvae challenged with ten of twelve strains belonging to the Harveyi clade of vibrios, and affected swimming motility in ten of the strains, biofilm formation in eight of the strains and mRNA levels of the AHPND toxin gens *pirAB* in one of the two AHPND-causing strains. These results indicate that indole-3-acetic acid could be an effective virulence inhibitor to control vibriosis in aquaculture.

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AUTHOR CONTRIBUTIONS

Shanshan Zhang: Conceptualization, methodology, investigation, validation, formal analysis, visualization, writing – original draft, funding acquisition; **Jana Van Haesebroeck:** Investigation, validation, formal analysis; **Qian Yang:** Supervision; **Tom Defoirdt:** Conceptualization, methodology, writing – review and editing, supervision, funding acquisition.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

REFERENCES

Austin, B. & Zhang, X.H. (2006). *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Letters in Applied Microbiology*, 43, 119-124.

Bassler, B.L., Greenberg, E.P. & Stevens, A.M. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi. Journal of Bacteriology*, 179, 4043-4045.

Berg, H.C. (1975). Chemotaxis in bacteria. *Annual Review of Biophysics and Bioengineering*, 4, 119-136.

Clatworthy, A.E., Pierson, E. & Hung, D.T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology*, 3, 541-548.

De Schryver, P., Defoirdt, T. & Sorgeloos, P. (2014). Early mortality syndrome outbreaks: a microbial management issue in shrimp farming? *PLoS Pathogens,* 10, e1003919.

Defoirdt, T., Sorgeloos, P. & Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, 14, 251-258.

Defoirdt, T., Bossier, P., Sorgeloos, P. & Verstraete, W. (2005). The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila, Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana. Environmental Microbiology*, 7, 1239-1247.

Defoirdt, T., Pande, G.S.J., Baruah, K. & Bossier, P. (2013). The apparent quorum-sensing inhibitory activity of pyrogallol is a side effect of peroxide production. *Antimicrobial Agents and Chemotherapy,* 57, 2870-2873.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W. & Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology,* 25, 472-479.

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W. & Bossier, P., 2006. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi, Vibrio campbellii,* and *Vibrio parahaemolyticus* isolates. *Applied and Environmental Microbiology,* 72, 6419-6423.

Dennis, P.G., Seymour, J., Kumbun, K. & Tyson, G.W. (2013). Diverse populations of lake water bacteria exhibit chemotaxis towards inorganic nutrients. *ISME Journal*, 7, 1661-1664.

Dong, X., Wang, H., Xie, G., Zou, P., Guo, C., Liang, Y. & Huang, J. (2017a). An isolate of *Vibrio campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease. *Emerging Microbes & infections*, 6, 1-3.

Dong, X., Bi, D., Wang, H., Zou, P., Xie, G., Wan, X., Yang, Q., Zhu, Y., Chen, M. & Guo, C. (2017b). pirAB vp-bearing *Vibrio parahaemolyticus* and *Vibrio campbellii* pathogens isolated from the same AHPND-affected pond possess highly similar pathogenic plasmids. *Frontiers in Microbiology,* 8, 1859.

Han, J.E., Tang, K.F., Tran, L.H. & Lightner, D.V. (2015a). Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus,* the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Diseases of Aquatic Organisms,* 113, 33-40.

Han, J.E., Tang, K.F., Pantoja, C.R., White, B.L. & Lightner, D.V. (2015b). qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus. Aquaculture*, 442, 12-15.

Josenhans, C. & Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *International Journal of Medical Microbiology,* 291, 605-614.

Karunasagar, I., Pai, R., Malathi, G. & Karunasagar, I. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi infection. Aquaculture,* 128, 203-209.

Kondo, H., Van, P.T., Dang, L.T. & Hirono, I. (2015). Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13. 17.5, isolated from diseased shrimp in Vietnam. *Genome Announcements,* 3, e00978-00915.

Kumar, V., Roy, S., Behera, B.K., Bossier, P. & Das, B.K. (2021). Acute hepatopancreatic necrosis disease (AHPND): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. *Toxins,* 13, 524.

Lee, C.-T., Chen, I.-T., Yang, Y.-T., Ko, T.-P., Huang, Y.-T., Huang, J.-Y., Huang, M.-F., Lin, S.-J., Chen, C.-Y. & Lin, S.-S. (2015a). The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proceedings of the National Academy of Sciences,* 112, 10798-10803.

Lee, J.-H., Wood, T.K. & Lee, J. (2015b). Roles of indole as an interspecies and interkingdom signaling molecule. *Trends in Microbiology,* 23, 707-718.

Lehmann, T., Hoffmann, M., Hentrich, M. & Pollmann, S., 2010. Indole-3-acetamide-dependent auxin biosynthesis: a widely distributed way of indole-3-acetic acid production? *European Journal of Cell Biology,* 89, 895-905.

Li, X., Yang, Q., Dierckens, K., Milton, D.L. & Defoirdt, T. (2014). RpoS and indole signaling control the virulence of *Vibrio anguillarum* towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *PloS One,* 9, e111801.

Liu, L., Xiao, J., Xia, X., Pan, Y., Yan, S. & Wang, Y. (2015). Draft genome sequence of *Vibrio owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. *Genome Announcements,* 3, e01395-01315.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods,* 25, 402-408.

Ljung, K., 2013. Auxin metabolism and homeostasis during plant development. *Development,* 140, 943-950.

Lu, Y. & Xu, J. (2015). Phytohormones in microalgae: a new opportunity for microalgal biotechnology? *Trends in Plant Science*, 20, 273-282.

Ludwig-Müller, J. (2015). Bacteria and fungi controlling plant growth by manipulating auxin: balance between development and defense. *Journal of Plant Physiology,* 172, 4-12.

Marques, A., Dinh, T., Ioakeimidis, C., Huys, G., Swings, J., Verstraete, W., Dhont, J., Sorgeloos, P. & Bossier, P. (2005). Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. *Applied and Environmental Microbiology,* 71, 4307-4317.

Mazur, H., Konop, A. & Synak, R. (2001). Indole-3-acetic acid in the culture medium of two axenic green microalgae. *Journal of Applied Phycology,* 13, 35-42.

Natrah, F.M., Bossier, P., Sorgeloos, P., Yusoff, F.M. & Defoirdt, T. (2014). Significance of microalgal–bacterial interactions for aquaculture. *Reviews in Aquaculture,* 6, 48-61.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O. & Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science,* 312, 436-439.

Novriadi, R., 2016. Vibriosis in aquaculture. *Omni-Akuatika*, 12, 1-12.

Prayitno, S.B. & Latchford, J. (1995). Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*. Effect of salinity and pH on infectiosity. *Aquaculture,* 132, 105-112.

Robertson, P., Calderon, J., Carrera, L., Stark, J., Zherdmant, M. & Austin, B. (1998). Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. *Diseases of Aquatic Organisms,* 32, 151-155.

Salini, R., Santhakumari, S., Ravi, A.V. & Pandian, S.K. (2019). Synergistic antibiofilm efficacy of undecanoic acid and auxins against quorum sensing mediated biofilm formation of luminescent *Vibrio harveyi. Aquaculture,* 498, 162-170.

Scott, H.M., Acuff, G., Bergeron, G., Bourassa, M.W., Gill, J., Graham, D.W., Kahn, L.H., Morley, P.S., Salois, M.J. & Simjee, S. (2019). Critically important antibiotics: criteria and approaches for measuring and reducing their use in food animal agriculture. *Annals of the New York Academy of Sciences,* 1441, 8-16.

Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M. & Morales-Covarrubias, M.S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (Litopenaeus vannamei) in Northwestern Mexico. *Applied and Environmental Microbiology,* 81, 1689-1699.

Spoel, S.H. & Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. *Cell Host & Microbe* 3, 348-351.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G.D., Djukić, S., Ćirković, I. & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS,* 115, 891-899.

Stirk, W.A., Ördög, V., Novák, O., Rolčík, J., Strnad, M., Bálint, P. & van Staden, J. (2013). Auxin and cytokinin relationships in 24 microalgal strains. *Journal of Phycology,* 49, 459-467.

Stocker, R., Seymour, J.R., Samadani, A., Hunt, D.E. & Polz, M.F. (2008). Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. *Proceedings of the National Academy of Sciences,* 105, 4209-4214.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K. & Lightner, D.V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms,* 105, 45-55.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P. & Verstraete, W. (1999). Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. *Applied and Environmental Microbiology*, 65, 2527-2533.

Yang, Q. & Defoirdt, T. (2015). Quorum sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi*. *Environmental Microbiology,* 17, 960-968.

Yang, Q., Pande, G.S.J., Wang, Z., Lin, B., Rubin, R.A., Vora, G.J. & Defoirdt, T. (2017). Indole signalling and (micro) algal auxins decrease the virulence of *Vibrio campbellii*, a major pathogen of aquatic organisms. *Environmental Microbiology,* 19, 1987-2004.

Zhang, S., Yang, Q. & Defoirdt, T. (2022a). Indole decreases the virulence of pathogenic vibrios belonging to the Harveyi clade. *Journal of Applied Microbiology,* 132, 167-176.

Zhang, S., Yang, Q., Fu, S., Janssen, C.R., Eggermont, M. & Defoirdt, T. (2022b). Indole decreases the virulence of the bivalve model pathogens *Vibrio tasmaniensis* LGP32 and *Vibrio crassostreae* J2-9. *Scientific Reports,* 12, 1-13.

Zhang, W., Yamane, H. & Chapman, D. (1993). The phytohormone profile of the red alga *Porphyra perforata*. *Botanica Marina,* 36, 257-266

Zhang, X.-H., Meaden, P. & Austin, B. (2001). Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi. Applied and Environmental Microbiology*, 67, 3161-3167.

Zorriehzahra, M. & Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as new emerging threat in shrimp industry. *Advances in Animal and Veterinary Sciences,* 3, 64-72.

TABLES

Table 1. Harveyi clade *Vibrio* strains used in this study.

Strain	Origin and/or synonyms	References
Vibrio parahaemolyticus		
M0904	AHPND-causing strain; isolated from the hepatopancreas of diseased shrimp (*Penaeus vannamei*), Mexico	Soto-Rodriguez et al., 2015
CAIM170	Isolated from the hemolymph of diseased shrimp (*Penaeus* spp.), Mexico	Defoirdt et al., 2006
V. harveyi		
VIB571	Isolated from sea bass (*Dicentrarchus labrax*), Spain	Zhang et al., 2001
VIB645	Isolated from sea bass (*Dicentrarchus labrax*), Tunisia	Zhang et al., 2001
E022	=STD3-101; isolated from diseased shrimp (*Penaeus vannamei*) larvae, Ecuador	Robertson et al., 1998
LMG22893	=CAIM148; isolated from the hemolymph of diseased shrimp (*Penaeus* spp.), Mexico	Defoirdt et al., 2006
V. campbellii		
S01	=20130629003S01; AHPND-causing strain; isolated from a shrimp (*Penaeus vannamei*) farm, China	Dong et al., 2017a
LMG21661	= CAIM415 = Z1; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	Defoirdt et al., 2006
LMG21362	= CAIM333 = M1; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	Defoirdt et al., 2006
LMG22888	= CAIM416 = Z2; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	Defoirdt et al., 2006
LMG22889	= CAIM417 = Z3; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	Defoirdt et al., 2006
LMG22890	= CAIM395 = STD3-131; isolated from diseased shrimp (*Litopenaeus* spp.) postlarvae, Ecuador	Defoirdt et al., 2006
BB120	= ATCC BAA-1116	Bassler et al., 1997

LMG, Laboratory of Microbiology Collection (Ghent University, Ghent, Belgium); CAIM, Collection of Aquacultural Important Micro-organisms (CIAD/ Mazatlan Unit for Aquaculture, Mazatlan, Mexico).

550 **Table 2.** Primers used in this study.

Gene	Primer sequences (5'-3')	References
pirA	TTG GACTGTCGAACCAAACG GCACCCCATTGGTATTGAATG	Han et al., 2015b
pirB	TGATGAAGTGATGGGTGCTC TGTAAGCGCCGTTTAACTCA	Han et al., 2015a
recA	GCTAGTAGAAAAAGCGGGTG GCAGGTGCTTCTGGTTGAG	Ma et al., 2015
rpoA	CGTAGCTGAAGGCAAAGATGA AAGCTGGAACATAACCACGA	Defoirdt et al., 2007b

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552 **Table 3.** Survival of brine shrimp (*Artemia franciscana*) larvae (%) after two days of challenge with
553 different *V. parahaemolyticus*, *V. harveyi* and *V. campbellii* strains, either or not treated with 200 µM
554 or 400 µM indole-3-acetic acid (IAA) (average ± standard deviation of three brine shrimp cultures).

Treatment	**Experiment 1**		**Experiment 2**	
	- IAA	**+ IAA (200 µM)**	**- IAA**	**+ IAA (400 µM)**
V. parahaemolyticus				
M0904	28 ± 5	48 ± 5 **	12±5	24±2 *
CAIM170	9 ± 7	13 ± 12	18±5	21±8
V. harveyi				
VIB571	54 ± 7	81 ± 5 **	49±8	71±5 *
VIB645	62 ± 7	51 ± 10	51±2	77±6 **
E022	59 ± 11	69 ± 11	61±7	54±8
LMG22893	57 ± 12	49 ± 5	66±8	91±7 *
V. campbellii				
S01	43 ± 7	54 ± 5	54±5	73±7 *
LMG21361	34 ± 4	52 ± 5 **	12±7	36±2 **
LMG21362	28 ± 5	40 ± 7	6±2	29±10 *
LMG22888	7 ± 3	19 ± 7	17±10	42±7 *
LMG22889	27 ± 3	44 ± 5 **	61±5	80±6 *
LMG22890	24 ± 5	44 ± 8 *	61±5	77±3 *

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556 The survival of unchallenged brine shrimp larvae was 76% ± 12% in the first experiment and 96% ± 2%
557 in the second experiment. Asterisks indicate significant differences in survival when compared to the
558 treatment with the same pathogen, but without indole-3-acetic acid (independent samples t-test); *: $P < 0.05$, **: $P < 0.01$.

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FIGURES

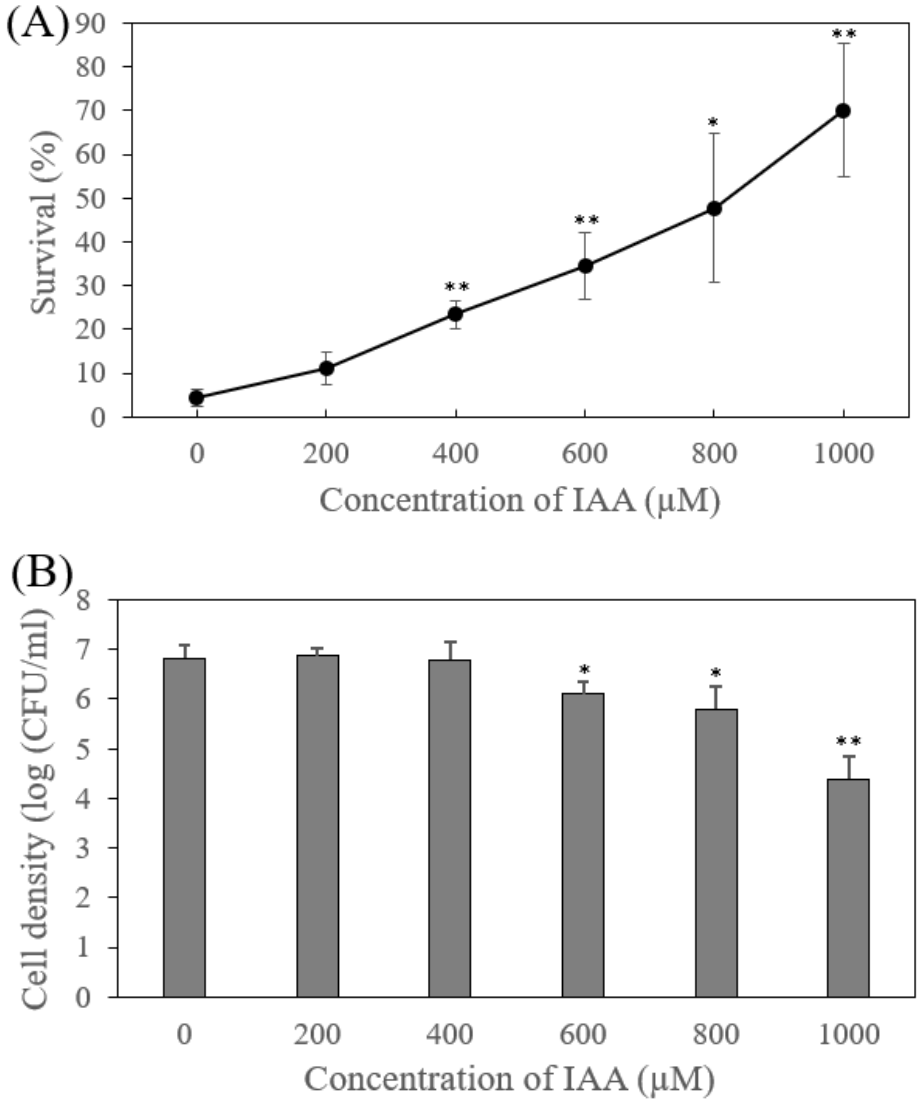


Figure 1. (A) Survival of brine shrimp (*Artemia franciscana*) larvae after two days of challenge with *Vibrio campbellii* BB120 and with different concentrations of indole-3-acetic acid (IAA). Indole-3-acetic acid was added to the brine shrimp rearing water at the start of the experiment. Error bars represent the standard deviation of three brine shrimp cultures. The survival of unchallenged larvae that were otherwise treated in the same way as challenged larvae was 98 ± 4%. **(B)** Density of *V. campbellii* BB120 in the gnotobiotic brine shrimp larval rearing water after two days of challenge. Asterisks indicate significant differences when compared with the treatment without indole-3-acetic acid (independent samples t-test); *: $P < 0.05$, **: $P < 0.01$.

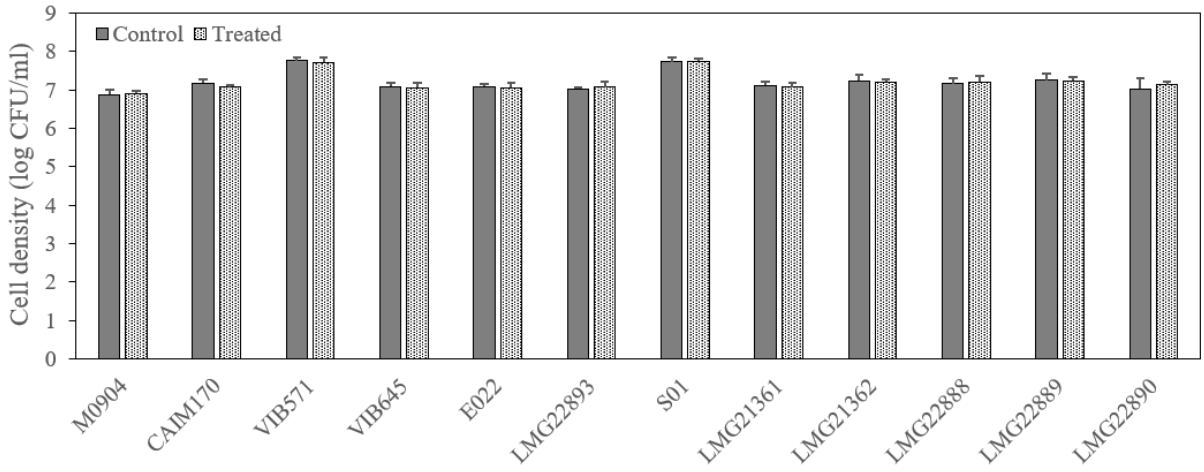


Figure 2. Density of the Harveyi clade *Vibrio* strains in the gnotobiotic brine shrimp larvae rearing water (with or without 400 µM indole-3-acetic acid) after 2 days of challenge.

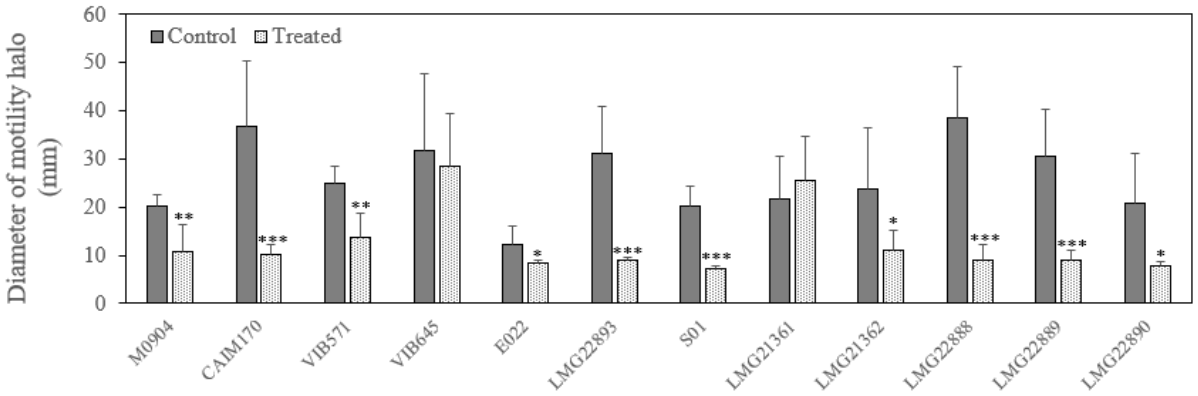


Figure 3. Impact of 200 µM indole-3-acetic acid on swimming motility of Harveyi clade *Vibrio* strains. The error bars represent the standard deviation of six replicate plates. Asterisks indicate significant differences when compared with the corresponding control without indole-3-acetic acid (Independent-Samples T-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

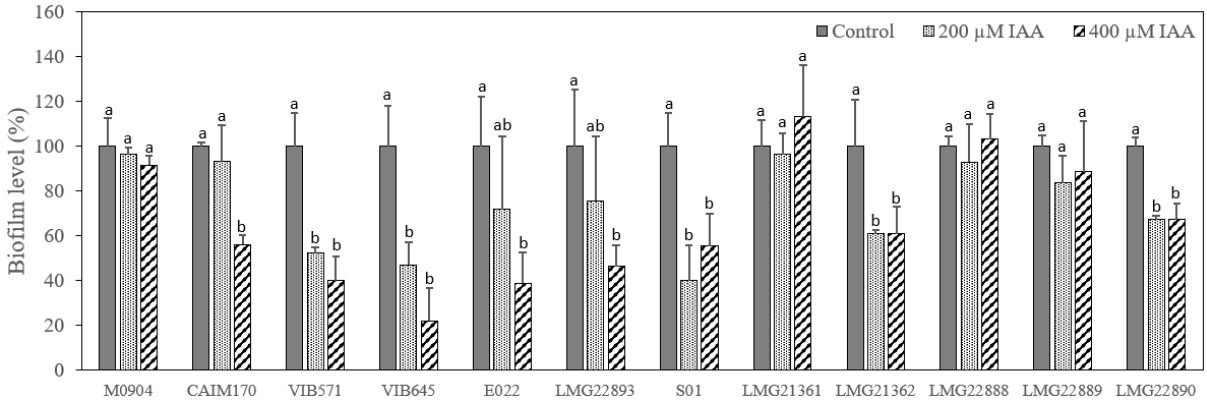
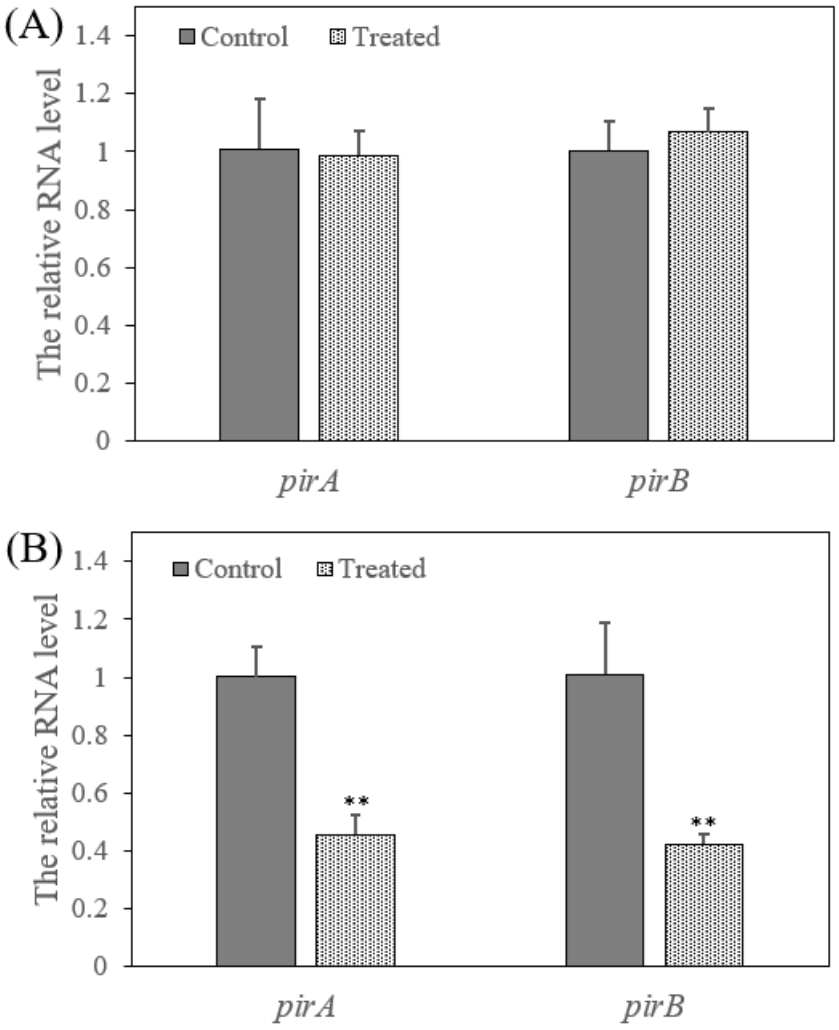


Figure 4. Impact of 200 µM and 400 µM indole-3-acetic acid (IAA) on biofilm formation of Harveyi clade *Vibrio* strains. The error bars represent the standard deviation of three independent experiments. For each *Vibrio* strain (each time comparing control, 200 µM indole-3-acetic acid and 400 µM indole-3-acetic acid), different letters indicate significant differences (One way ANOVA with Tukey's post hoc test; $P<0.05$).



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663 **Figure 5.** Effect of 400μM indole-3-acetic acid on the mRNA levels of the *pirAB* toxin genes in *V.*
664 *campbellii* S01 (A) and *V. parahaemolyticus* M0904 (B). Strains were grown in LB_{35} medium without
665 or with 400 μM indole-3-acetic acid for 6 hours. Asterisks indicate significant differences when
666 compared with the corresponding control without indole-3-acetic acid (Independent-samples T-test; * P
667 < 0.05; ** P < 0.01; *** P < 0.001).

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