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Original Article

Impact of a *Vibrio parahaemolyticus* Strain Carrying a Quorum Sensing Inhibiting Gene Construct on the Vitality and Resistance of Whiteleg Shrimp to the Wild-type Strain

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Abstract: *Vibrio parahaemolyticus* is a widespread pathogen of marine aquaculture animals, especially whiteleg shrimp (*Litopenaeus vannamei*), with mortality rates of up to 100%, causing substantial economic losses to the marine aquaculture industries. Traditional treatments, including antibiotics and chemicals, are becoming ineffective due to emerging bacterial resistance and concerns related to environmental contamination and human health. Thus, novel approaches for the treatment and prevention of diseases caused by *Vibrio* bacteria are needed. A recent new and promising approach is to inhibit quorum sensing, which is the regulation of gene expression and thus cell behaviors in response to fluctuations in cell-population density. In this study, we explored whether a *V. parahaemolyticus* strain with inhibited quorum sensing could express a reduced pathogenicity level and stimulate better resistance in whiteleg shrimp. The strain was *V. parahaemolyticus* PBN, which was created in a previous study and harbored a plasmid (pCR2.1) inserted with the gene *qrr_vibrio*, containing the preservative sequences of quorum-sensing regulator sRNAs (*qrr*) in *Vibrio* bacteria. This strain exhibited a diminished capacity for biofilm formation compared to its wild-type counterpart. Furthermore, shrimp infected with PBN demonstrated 20-25% higher survival rates than those exposed to the wild-type strain. We further investigated the impact of various cell densities of PBN on shrimp resistance and found that the resistance of shrimp to the wild-type was enhanced if shrimp were pretreated with higher densities of PBN, particularly at 2×10^8 and 4×10^8 CFU/mL. The results are interesting and strongly support the interruption of quorum sensing as an effective approach to control diseases caused by *Vibiro* bacteria.

Keywords: Litopenaeus vannamei, Vibrio parahaemolyticus, quorum sensing, qrr_vibrio.

1. Introduction

Vibrio species are significant pathogens in aquatic environments, posing a substantial threat to the health of various aquatic animals. Moreover, climate change with rising sea surface temperatures exacerbates the *Vibrio* infection and diminishes the seafood production globally. *Vibrio parahaemolyticus*, a member of the genus *Vibrio*, is Gram-negative, characterized by its curved, rod-shaped

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morphology, and commonly found in brackish and saltwater environments. *V. parahaemolyticus* is known to cause acute hepatopancreatic necrosis disease (AHPND) in shrimp, a severe condition that can lead to significant mortality [1]. Losses due to AHPND in 2015 were determined to be 26 million USD [2]. In Vietnam, AHPND was first recorded in 2011, and spread to about 59. 000 ha of farms in the Mekong Delta [3]. The pathogenesis of *V. parahaemolyticus* involves attachment to the liver surface of shrimp, followed by the formation of biofilms, which provide protection against antibiotics, disinfectants, and other treatment modalities.

Traditional treatments to control *V. parahaemolyticus*, including the uses of antibiotics and chemicals, are becoming ineffective due to emerging bacterial resistance, along with concerns related to environmental contamination and human health. Thus, novel approaches for the treatment and prevention of diseases caused by *Vibrio* bacteria are needed. Quorum sensing (QS) suppression, a novel strategy that targets bacterial communication mechanisms, has emerged as a promising approach for controlling *Vibrio* epidemics. QS inhibition offers several advantages over conventional treatments, as it disrupts bacterial communication without causing harm to living cells and thus stimulates the development of bacterial resistance to lesser degrees.

Quorum sensing (QS) is a sophisticated form of bacterial communication that relies on the production and release of autoinducer (AI) signaling molecules [4]. These AI signals are recognized by neighboring bacteria, enabling coordinated gene expression within a population [5]. At low cell densities, AI production is minimal, leading to the expression of small regulatory RNAs (sRNAs) known as Qrr(s). These Qrrs regulate the expression of various genes involved in processes such as biofilm formation, luminescence, swarming motility, and antibiotic resistance. Conversely, at high cell densities, AI production is significantly increased, resulting in reduced Qrr expression and the activation of virulence factors, including toxins. Therefore, *qrr* genes, which encode Qrrs, offer a promising target for designing and controlling QS. Our previous research successfully designed and constructed a *qrr_vibrio* gene, consisting of 133 nucleotides, based on the conserved sequences of the *qrr* genes of *Vibrio* species [6]. The *V. parahaemolyticus* strain carrying the *qrr_vibrio* gene, denoted as strain PBN, was shown to have much reduced mobility compared to the wild-type strain in previous research [7]. Strain PBN is expected to exhibit reduced virulence when infecting whiteleg shrimp, potentially enhancing the shrimp's resistance to *V. parahaemolyticus* infections.

This study investigated the biofilm forming capacity of both the wild-type and the recombinant *V. parahaemolyticus* PBN strains. In vivo experiments were conducted to assess the resistance of whiteleg shrimp (*Litopenaeus vannamei*) to infection with the wild-type strains after pretreated with varying cell densities of PBN.

2. Experimental

2.1. Bacterial Strains and Culture Conditions

This study utilized three *Vibrio parahaemolyticus* strains: PAM17, a wild-type strain (VP), a strain carrying the vector pCR2.1 plasmid and thus having no *qrr_vibrio* gene (VP(-)), and a recombinant strain containing the pCR2.1_QRR plasmid inserted with the *qrr_vibrio* gene (PBN), created in a previous study [7]. These strains were provided by the GREENLAB laboratory (Center for Life Science Research, Faculty of Biology, VNU University of Science). Whiteleg shrimp (*Litopenaeus vannamei*) was purchased from reliable sources in Thuong Dinh market, Thanh Xuan ward, Ha Noi. Strain VP was cultured in 3% Luria-Bertani (LB) broth, while the VP(-) and PBN strains were cultured in 3% LB broth supplemented with ampicillin (Amp, 100 μg/mL). All bacterial strains were incubated at 37 °C.

2.2. Biofilm Formation Measurement

The biofilm formation capabilities of the three bacterial strains (VP, VP(-), and PBN) were assessed using a standard biofilm assay, according to Thuy et al., (2017) [7]. Bacterial cultures were grown in 3% Luria-Bertani (LB) medium supplemented with a 1:1 ratio of seasalt water at 37 °C with shaking at 200 rpm overnight until a final concentration of approximately 10^8 CFU/mL was achieved. Subsequently, 500 µL of each bacterial culture was transferred to individual 2-mL Eppendorf tubes and incubated without shaking at 37 °C for 48 hours to allow biofilm formation. After incubation, the supernatant was carefully removed, and the remaining cells of the biofilm attached to the tube surface were washed twice with 1× phosphate-buffered saline (PBS) buffer to remove any unbound cells. Finally, 500 µL of distilled water was added to each Eppendorf tube, the contents were vortexed, and the optical density (OD) at 600 nm was measured to quantify the amount of biofilm formed.

2.3. The Shrimp Infecting Experiment to Test the Pathogenicity of the qrr_vibrio Carrying Strain PBN

Adult shrimp with a size of 0.5 g - 1.5 g were pre-infected with PBN cells at a density of 4×10^8 CFU/mL for 2 days (200 shrimp/4 tanks). Similarly, for the control, shrimp were also reared but not in contact with any bacteria (200 shrimp/4 tanks). During this time, we monitored and removed dead shrimp. Surviving shrimp were subsequently cultured and infected with VP, VP(-), and PBN cells at a density of 4×10^8CFU/mL on day 3. The experimental tanks and the oxygen aerator connecting tubes were disinfected using alcohol, and the oxygen aerators were sterilized to prevent cross-contamination. Each tank was also equipped with covers to protect against external contamination. Shrimp were reared in the laboratory at room temperature 25±2 ^{o}C, and fed once (with the feed amount equivalent to 5-8% of shrimp body weight) per day, while water was not changed and dirt was not removed by vacuuming. The number of shrimp was monitored and dead shrimp were removed daily. The experiment is summarized by the diagram below (Figure 1). The data were statistically analyzed by independent samples T-test with a 95% confidence level.

2.4. Experiment to Infect Shrimp with Different Cell Densities of PBN to Evaluate the Ability to Stimulate Shrimp Resistance

Adult whiteleg shrimp, with sizes ranging from 0.5 to 1.5 g, were randomly assigned to six experimental groups (100 shrimp/tank). Five groups were reared with strain PBN supplemented into the rearing water at varying cell densities: 10^6, 2×10^7, 4×10^7, 2×10^8, and 4×10^8 CFU/mL. A sixth group served as a control and was maintained without contacting any bacteria. After two days, the number of surviving shrimp in each group was recorded.

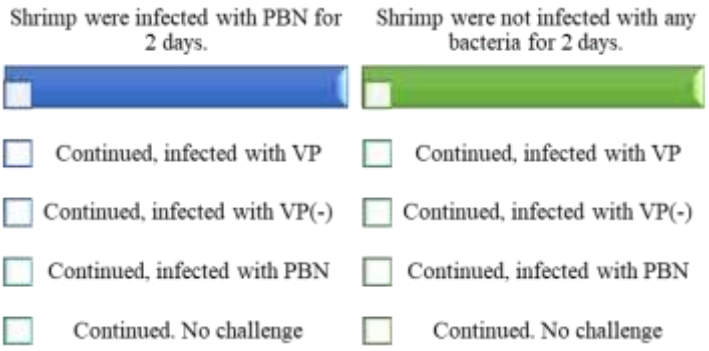

Figure 1. Diagram of shrimp rearing experiment to test the pathogenicity of the *qrr_vibrio* carrying strain PBN.

Surviving shrimp were then randomly divided into two subgroups: one subgroup continued to be cultured with the wild-type *V. parahaemolyticus* strain at a density of 4×10^8CFU/mL, while the other subgroup served as a control and was left unchallenged. The devices utilized in this experiment were prepared and disinfected following the procedures outlined in section 2.3. All shrimp were maintained in laboratory conditions at room temperature (25±2 ^oC) and fed once daily (with the feed amount equivalent to 5-8% of shrimp's body weight). Water changes and vacuuming of debris were not performed during the experiment. Mortality was monitored daily, and dead shrimp were removed. The experimental design is summarized in Figure 2. The data were statistically analyzed by independent samples T-test with a 95% confidence level.

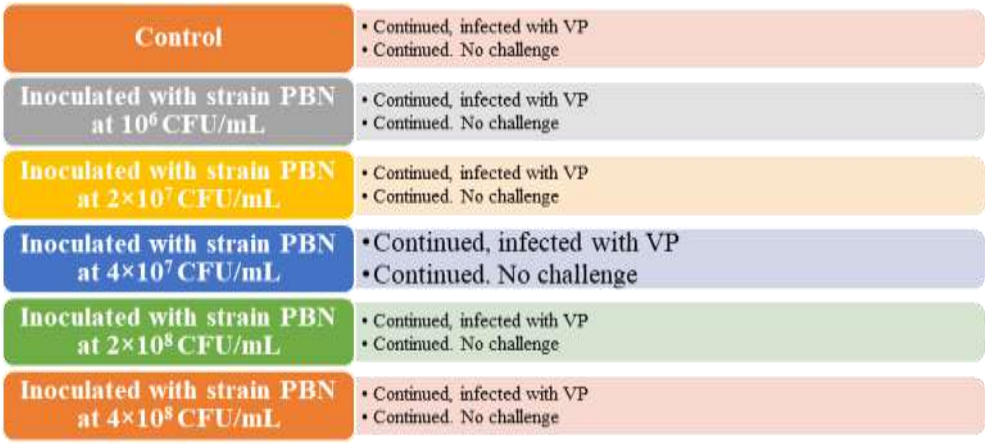

Figure 2. Experimental design diagram to evaluate the ability to stimulate shrimp resistance when cultured with different cell densities of PBN.

3. Results and Discussion

3.1. Ability to Form Biofilm of the Bacterial Strains

Biofilm formation in *Vibrio parahaemolyticus*, as well as in other bacterial species, is characterized by the accumulation of high-density bacterial populations embedded within a matrix of extracellular polymeric substances (EPS). These EPS, produced by the bacteria themselves, facilitate adhesion to both biotic and abiotic surfaces [8]. Biofilm formation is therefore essential in the pathogenicity of pathogenic bacteria, including vibrios [9].

Biofilm formation assay results (Figure 3) revealed a comparable level of biofilm production in the *V. parahaemolyticus* strain carrying only the pCR2.1 vector (VP(-), OD600 nm = 0.2 ± 0.0229) and the wild-type strain (VP, OD600 nm = 0.1836 ± 0.0225).

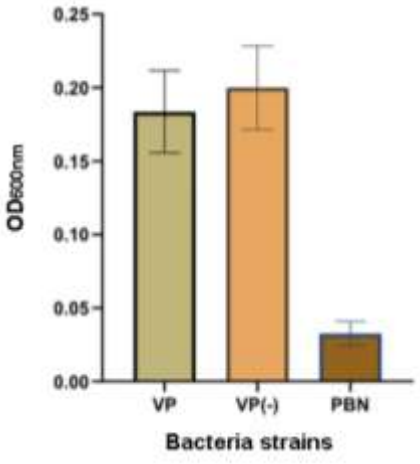

Figure 3. The biofilm formation abilities of the tested bacterial strains as demonstrated by the optical densities of biofilm-forming cells at 600 nm.

In contrast, the recombinant strain PBN, harboring the QS-inhibiting gene, exhibited significantly reduced biofilm formation (OD600 nm = 0.0326 ± 0.0067), approximately 6-7 times lower than the VP and VP(-) strains.

These results strongly suggest that the *qrr_vibrio* gene expressed in PBN effectively disrupts quorum sensing (QS) pathways, leading to a substantial reduction in biofilm formation compared to the wild-type strain. Furthermore, the introduction of the pCR2.1 vector alone (VP(-)) did not significantly alter QS activity in *V. parahaemolyticus*, indicating that the observed reduction in biofilm formation in strain PBN is specifically attributed to the presence of the *qrr* gene. Based on these results, it is hypothesized that the disruption of QS pathways by the *qrr* gene may also lead to a reduction in the virulence potential of PBN. This finding is consistent with our previous research on strain PBN [7]. This strain not only demonstrated a diminished capacity to develop robust biofilm but also exhibited reduced motility - both of which are phenotypic signatures indicative of QS inhibition [7].

There have been many studies on the mechanism of QS inhibitors that prevent biofilm formation such as targeting the AI signaling molecules [10], targeting the signaling molecule receptors [11], blocking the signaling cascade [12]. Our research group has suggested a novel approach by targeting *qrr* genes, which encode quorum-sensing regulator RNAs. This represents a unique and potentially impactful strategy, as the Qrrs play a critical role in the post-transcriptional regulation of key QS components.

3.2. Evaluation of the Pathogenicity of Quorum Sensing-inhibited Vibrio Parahaemolyticus in Whiteleg Shrimp (Litopenaeus Vannamei)

The results of the survival experiment (Figure 4) demonstrated significant differences in mortality rates among the various treatment groups after five days. The highest mortality rates were observed in the tanks inoculated with VP(-) (*V. parahaemolyticus* strain carrying the empty pCR2.1 vector) and the wild-type strain VP, with mortality reaching approximately 70-80%. This represents a five-fold increase in mortality compared to the control group, which exhibited significantly lower mortality rates ($p<0.05$). Tanks inoculated with strain PBN (carrying the *qrr_vibrio* gene) exhibited an intermediate mortality rate of approximately 55-60%.

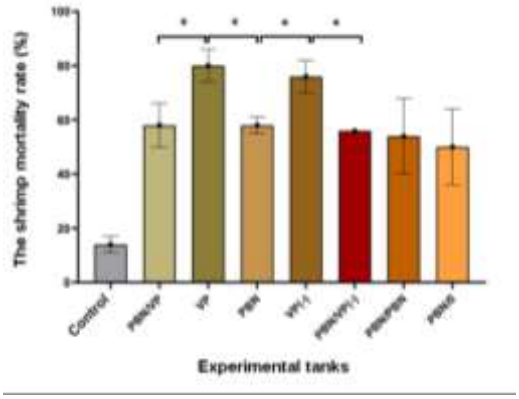

Figure 4. The mortality rates of whiteleg shrimp after five days of exposure to different *Vibrio parahaemolyticus* strains.

Note: Control: shrimp maintained in tanks without any bacteria. VP: shrimp exposed to the wild-type *V. parahaemolyticus* strain (VP). VP(-): shrimp exposed to the *V. parahaemolyticus* strain carrying the pCR2.1 vector (VP(-)). PBN: shrimp exposed to the recombinant *V. parahaemolyticus* strain carrying the plasmid inserted with the *qrr_vibiro* gene (PBN). PBN/VP: shrimp pre-infected with strain PBN and subsequently exposed to strain VP. PBN/VP(-): shrimp pre-infected with strain PBN and subsequently exposed to strain VP(-). PBN/PBN: shrimp pre-infected with strain PBN and subsequently exposed to strain PBN. PBN/0: shrimp pre-infected with strain PBN and subsequently maintained without any further bacterial inoculation. *: statistical analysis using a 95% confidence level was performed to identify significant differences ($p < 0.05$) in mortality

rates between treatment groups. The experiment was replicated twice.

The observed reduction in mortality rates in shrimp pre-infected with strain PBN, followed by exposure to various *V. parahaemolyticus* strains, suggests a potential enhancement of shrimp resistance. This observation supports the hypothesis that pre-exposure to PBN, carrying the *qrr_vibrio* gene, stimulates a protective response in shrimp, reducing the impact of subsequent exposure to highly virulent *V. parahaemolyticus* strains. However, the relatively high mortality rate observed in the PBN experimental group alone indicates that further investigation is warranted to fully understand the pathogenicity of the recombinant *V. parahaemolyticus* strain carrying the *qrr_vibrio* gene.

The lower mortality rate observed in shrimp exposed to strain PBN compared to strain VP aligns with the hypothesis that the *qrr_vibrio* gene, by disrupting quorum sensing (QS) pathways, reduces the virulence of *V. parahaemolyticus*. This observation is consistent with the findings mentioned above, which demonstrated a significant reduction in biofilm formation ability of PBN compared to the wild-type strain VP. The *qrr_vibrio* gene may modulate QS regulation, leading to enhanced shrimp resistance when reared in an environment containing the bacteria carrying it.

3.3. Evaluation of the Protective Efficacy of Various Pre-infecting Cell Densities of PBN against the Wild-type Vibrio Parahaemolyticus in Whiteleg Shrimp

The results of the experiment testing the effect of 5-day exposure to varying cell densities of PBN (Figure 5) on the mortality rate of whiteleg shrimp are interesting. The control group (uninfected) exhibited significantly lower mortality rates compared to the group directly exposed to the wild-type strain VP (control/VP), which experienced a mortality rate of approximately 70-80%, nearly three times higher than the control group.

In the groups pre-infected with strain PBN and subsequently exposed to strain VP (PBN/VP), a significant variation in mortality was observed depending on the initial cell density of PBN. The highest mortality rate (68%) was observed in the group pre-infected with the lowest PBN density (10^6CFU/mL), while the lowest mortality rate (40%) was observed in the group pre-infected with the highest PBN density (4×10^8 CFU/mL).

Statistical analysis revealed a significant difference ($p < 0.05$) in mortality rates between the control/VP group and the PBN/VP groups pre-infected with PBN densities of 2×10^8 and 4×10^8 CFU/mL. This suggests that pre-exposure to strain PBN at higher densities significantly reduces the mortality rate upon subsequent exposure to the highly pathogenic wild-type VP.

These findings provide evidence that pre-infection with strain PBN, carrying the *qrr_vibrio* gene, can stimulate a protective response in shrimp, enhancing their resistance to *V. parahaemolyticus* infection. The more cells of PBN were in contact with shrimp, the stronger protective response was expressed in shrimp. This protective effect is evident in the reduced mortality rates observed in the PBN/VP groups, particularly at higher cell densities of PBN. The observed protective effect of strain PBN is analogous to a "vaccine" effect, where pre-exposure to a less virulent strain (PBN) enhances the host's resistance to subsequent infection with a more virulent strain (VP).

The mortality rates in the PBN/0 groups (pre-infected with PBN and subsequently maintained without further bacterial inoculation) varied depending on the initial PBN density. The highest mortality rate (over 70%) was observed in the group pre-infected with a PBN density of 4×10^7 CFU/mL, while the lowest mortality rate (approximately 45%) was observed in the groups pre-infected with PBN densities of 2×10^8 and 4×10^8 CFU/mL.

These findings suggest that pre-exposure to lower PBN densities (0 to 4×10^7 CFU/mL) resulted in higher mortality rates in the PBN/0 groups compared to the PBN/VP groups. However, at higher PBN densities (2×10^8 to 4×10^8 CFU/mL), the mortality rates in the PBN/0 and PBN/VP groups were comparable, ranging from 40-50%. Furthermore, a trend of decreasing difference in mortality rates between the PBN/0 and PBN/VP groups was observed with increasing PBN densities.

Despite this trend, statistical analysis revealed no significant difference ($p > 0.05$) in mortality rates between the PBN/VP groups at different PBN densities. This suggests that, while pre-exposure to lower cell densities of PBN may result in a higher mortality rate in the absence of subsequent VP exposure, pre-exposure to higher cell densities of PBN effectively neutralizes the virulence of strain VP, leading to similar mortality rates in both the PBN/0 and PBN/VP groups.

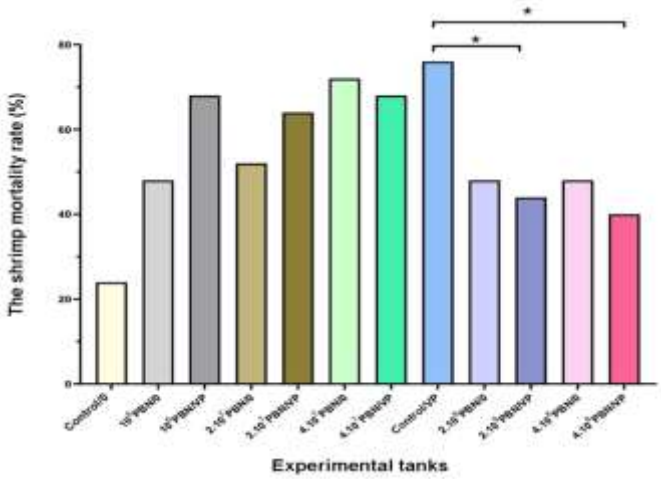

Figure 5. The mortality rates of whiteleg shrimp after five days of exposure to varying cell densities of strain PBN.

Note: Control/0: shrimp maintained in tanks without any bacteria. 10^6 PBN/0, 2×10^7 PBN/0, 4×10^7 PBN/0, 2×10^8 PBN/0, and 4×10^8 PBN/0: shrimp pre-infected with strain PBN at a cell density of 10^6, 2×10^7, 4×10^7, 2×10^8, or 4×10^8 CFU/mL, respectively, and then not contacting with any bacteria. Control/VP: shrimp maintained in tanks with VP. 10^6 PBN/VP, 2×10^7 PBN/VP, 4×10^7 PBN/VP, 2×10^8 PBN/VP, and 4×10^8 PBN/VP: shrimp pre-infected with strain PBN at a cell density of 10^6, 2×10^7, 4×10^7, 2×10^8, and 4×10^8 CFU/mL, respectively and then infected with VP. *: statistical analysis using a 95% confidence level was performed to identify significant differences ($p < 0.05$) in mortality rates between treatment groups.

These findings highlight the importance of the cell density of PBN in determining its protective efficacy against *V. parahaemolyticus* infection. While lower PBN densities may not provide sufficient protection, higher densities appear to effectively mitigate the virulence of the wild-type strain, confirming a potential for PBN as a "vaccine-like" biocontrol agent in shrimp aquaculture.

Recent treatments for vibriosis commonly involve the use of probiotics, which consist of beneficial microorganisms that can serve as nutritional supplements, promote the growth of beneficial bacteria, and inhibit the proliferation of harmful bacteria. While the efficacy of probiotics in managing vibriosis has been documented, there are controversial debate

among researchers regarding their use [14]. Certain components in probiotics may enhance antibiotic resistance, raising concerns about their application in shrimp aquaculture [14]. In contrast, quorum sensing strategy exerts a selective and less-intensive pressure on bacterial populations, contributing to a gradual reduction in bacterial growth rates. By disrupting the communication mechanisms that bacteria use to coordinate virulence, quorum sensing inhibition presents a promising alternative for treating diseases in aquaculture.

Although many studies have tried to use *V. parahaemolyticus* antigens to develop vaccines against this disease, there is no commercial vaccine that works against AHPND for shrimp now [13, 14]. Nakamura and colleagues reported the most up-to-date study on the use of the egg yolk immunoglobulin IgY against recombinant PirA and PirB toxins (produced by using the toxin genes *pirA* and *pirB* in *V.parahaemolyticus*) in feed and showed passive immunity against AHPND [15] but this product has not been valorized. On the other hand, producing antibodies is costly and complicating while it is ineffective to use a huge amount of antibodies in feed spreading in shrimp ponds. Moreover, the effectiveness of antibodies is always challenged by the constant evolution of bacteria. Notably, there were studies on deletion of genes responsible for the virulence of pathogenic bacteria to develop live attenuated vaccines against *V. parahaemolyticus* [16]. This approach could be effective due to the prevention of genes from expressing and controlling virulent factors. However, the mechanism for virulence in bacteria has been not understood completely and need to be thoroughly investigated. In our study, the recombinant strain PBN could also be used as an "live attenuated vaccine-like" agent. Our approach aims to decrease both toxicity and growth of the bacteria, thereby restricting the spread of pathogens. Thus, our study represented a different and new effort to develop potential vaccine against vibriosis for shrimp aquaculture industry.

4. Conclusion

The results of this study demonstrated that shrimp pre-infected with a *V. parahaemolyticus* strain that strongly expresses quorum sensing regulator RNA genes (PBN) exhibited significantly higher survival rates compared to those infected with the wild-type strain (VP). Furthermore, pre-exposure to PBN at densities ranging from 2×10^8 to 4×10^8 CFU/mL effectively stimulated shrimp resistance, leading to increased survival rates upon subsequent exposure to the highly pathogenic strain VP. These findings suggest that strain PBN possesses the potential to be utilized as an immunostimulant in shrimp aquaculture.

However, the current study was conducted under controlled laboratory conditions with a limited sample size and duration. Further investigations at higher scales with more extended durations and under real-world production settings are necessary to validate these findings and optimize the application of strain PBN. Future studies should focus on determining the optimal PBN density for maximum protective efficacy and on investigating the specific mechanisms underlying the observed immunostimulatory effect.

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