INTRODUCTION

The outbreak of vibriosis diseases has a negative effect on the sustainable development of the aquaculture industry and also causes the economic loss to aquaculture industry (Baker-Austin et al., 2018; De Schryver et al., 2014; Stentiford et al., 2017). Vibrio parahaemolyticus, Vibrio anguillarum, Vibrio alginolyticus and Vibrio harveyi are the most common pathogens for aquatic animals, in which V. parahaemolyticus has been frequently involved in disease outbreaks worldwide (Baker-Austin et al., 2018; Bauer et al., 2021; Hickey & Lee, 2017; Mohamad et al., 2019). V. parahaemolyticus is a Gram-negative bacterium in various aquatic environments including marine, coastal and freshwater, which could cause infectious diseases in oysters, shrimp and fish (Johnson...
et al., 2012; McCarter, 1999; Yang et al., 2019). The infection of *V. parahaemolyticus* could cause the external haemorrhages and tail rot in fish, the white spots on foot of abalones and the acute hepatopancreatic necrosis disease in shrimp (Alcaide et al., 1999; Cai et al., 2007; Imaizumi et al., 2018). Furthermore, *V. parahaemolyticus* is also an important foodborne pathogen in humans, which could cause the acute gastroenteritis with diarrhoeal by the consumption of contaminated raw seafood (Soumaré et al., 2007). Therefore, the contamination of *V. parahaemolyticus* in aquatic products should be taken a great concern due to its negative effects on the aquatic production and public health.

In 1996, *V. parahaemolyticus* serotype O3:K6 was identified in India for the first time, which was then frequently reported to cause diarrhoeal outbreaks in humans worldwide (Okuda et al., 1997; Velazquez-Roman et al., 2014). *Vibrio parahaemolyticus* serotypes O3:K6 has been known as the most prevalent serotype in the clinical samples, which carries *tdh* and/or *trh* genes as the main virulence factors (Guerrero et al., 2017; Raghunath, 2015). *Vibrio parahaemolyticus* serotype O3:K6 has been detected in 56.8% of fish samples and 26.3% of clinical samples in India, respectively (Guin et al., 2019). In Mexico, *V. parahaemolyticus* was detected in 15% of sea water, oyster and fish samples from 2001 to 2002 (Cabrera-García et al., 2004). *Vibrio parahaemolyticus* has been reported in 16.2% of freshwater fish samples and 42.0% of seafood samples in China, while 80.7%, 15.0% and 4.3% of shellfish, crustaceans and fish samples were contaminated by *V. parahaemolyticus*, respectively (Chen et al., 2021; Su & Chen, 2020). Thus, the continuous monitoring of *V. parahaemolyticus* in aquatic animals and products is important for the aquatic food safety.

The dietary acidifiers were applied in the feed to improve the growth, survival and feed utilization of aquatic animals (Asriqah et al., 2018). Previous studies showed that the supplement of citric acid, sorbic acid or formic acid in feed could increase the bioavailability of minerals in rainbow trout and Pacific white shrimp (He et al., 2017; Vielma & Lall, 1997). Moreover, short-chain organic acids could also provide energy for renewing the intestinal epithelia and maintaining the gut health (Abu Ela & Ragaa, 2015). However, the broodstock and fresh feed were identified as the important sources of *V. parahaemolyticus* (Yingkajorn et al., 2014). Previous studies demonstrated that the preadaptation of pathogens to sub-lethal acidic conditions could increase the bacterial survival ability under lethal acidic conditions (Cakar et al., 2019; Kuper & Jung, 2005; Lee et al., 2007). This phenomenon is known as the acid tolerance response (ATR). Additionally, ATR has been related to the enhancement of bacterial virulence (Merrell & Camilli, 1999; O’Driscoll et al., 1996). The above observations have indicated that the acidic feed may increase the virulence and survival ability of pathogens, which may increase the risk of *V. parahaemolyticus* causing infectious diseases in both aquatic animals and humans.

*Vibrio parahaemolyticus* is an important gastrointestinal pathogen, which could colonize in the small intestine of the host. A previous study indicated that CadBA is responsible for ATR in *V. parahaemolyticus* (Tanaka et al., 2008). This study aimed to assess how acid influences the virulence and survival ability of *V. parahaemolyticus*. Genes involved in ATR were identified by RNA-seq, and the role of CadC in the modulation of the ATR was further investigated. Our study also demonstrated how sub-lethal acidic conditions could affect the motility and cytotoxicity of *V. parahaemolyticus*.

## MATERIALS AND METHODS

### 2.1 Bacterial strains, plasmids and culture conditions

The strains, plasmids and primers used in this study are listed in Tables 1 and 2. The *V. parahaemolyticus* RIMD2210633 strain and derivatives (mutants and complementary) were grown at 37°C in Luria–Bertani (LB) medium with 3% NaCl, and the *Escherichia coli* strains were grown at 37°C in LB medium.

### 2.2 Construction of the deletion mutant and complementary strains

The deletion mutant strains of *V. parahaemolyticus* *cadA, cadB* and *cadC* were constructed as previously described (Zhou et al., 2010). Briefly, the upstream and downstream fragments of the *cadA, cadB* and *cadC* genes were amplified using PCR with specific primers (Table 2) and cloned into the suicide plasmid pDM4. The recombinant plasmids were transformed into *E. coli* SM10 *ipir*, conjugated into the WT and then selected in the LB medium with carbenicillin and chloramphenicol. The second cross-over recombination products were selected in LB medium with 10% sucrose. The mutant strains were verified using PCR with specific primers (*cadA-out-F/R, cadB-out-F/R, cadC-out-F/R*), followed by sequencing.

The ribosome-binding site (RBS) and open reading frame (ORF) regions of *cadC* were amplified using PCR with specific primers (Table 2) and cloned into the pMMB207 plasmid. The recombinant plasmid pMMB207-*cadC* was transformed into *E. coli* SM10 *ipir* and conjugated to the Δ*cadC*. Polymerase chain reaction and sequencing confirmed the complementary strain, which was named as *cadC*.

### 2.3 ATR analysis

The overnight cultured strains WT, Δ*cadA*, Δ*cadB*, Δ*cadC* and *cadC* were diluted into fresh LB medium and incubated at 37°C until they reached an optical density (OD$_{600}$) of 0.2–0.3. The strains were resuspended in LB medium at different pH values (pH 7.5 or pH 5.5) and cultured at 37°C for 1 hr. Subsequently, the strains cultured at pH 7.5 or pH 5.5 LB medium were transferred into LB medium at pH...
4. An aliquot was taken from each culture at the indicated time points and diluted appropriately. The bacteria were placed onto the LB agar plates, and the surviving bacteria were counted. Each experiment was repeated 3 times.

2.4 | RNA-sequencing analysis

The WT strain was cultured overnight and then diluted 1:100 into fresh LB medium. After culturing for 3 hr, half of the culture was treated with LB medium at pH 5.5 (HCl) for 2 hr. The remaining bacteria were further cultured during this period. One millilitre of bacterial cells was collected; their total RNA was extracted using an RNA Easy kit (Qiagen). The RNA samples were treated with DNase I (Invitrogen) to remove any genomic DNA (gDNA) contamination and sequenced by the Illumina HiSeq (GENEWIZ). The quality control and filtered data were then aligned to the reference genome via software Bowtie 2 version 2.2.6 (Langmead & Salzberg, 2012). HTSeq version 0.6.1p1 was used to estimate gene expression levels from the clean data (Anders et al., 2015). The different expression analysis was conducted using the DESeq 2 Bioconductor package (Love et al., 2014).

2.5 | Quantitative real-time reverse transcription PCR (qRT-PCR)

The WT strain was cultured and exposed to sub-lethal acidic pH as described above in RNA-sequencing analysis. One mL of bacterial cells from each of the exposed and unexposed cultures was collected; their total RNA was extracted using the RNA Easy kit (Qiagen). The total RNA samples were treated with DNase I to remove gDNA. Equal amounts of RNA (1 μg) were used to generate the first-strand complementary DNA (cDNA) (Takara) using
random primers. Three independent qRT-PCR experiments were performed with specific primer pairs listed in Table 2. The reaction mixture was run on the Applied Biosystems 7500 Real-Time System (Applied Biosystems). The messenger RNA (mRNA) levels were normalized to the \( \text{gyrB} \) mRNA level in each sample using the \( 2^{-\Delta\Delta C_t} \) method.

### 2.6 Electrophoretic mobility shift assay

The DNA-binding domain of cadC was amplified using PCR (Table 2), cloned into the pET28a plasmid and purified the CadC-his protein. Electrophoretic mobility shift assay (EMSA) analysis was performed as previously described (Gu et al., 2016). The DNA probes were amplified and purified by a DNA Gel Purification Kit (TIANGEN). The EMSA reaction mixture (20 \( \mu \)l) was mixed with 10 ng of the DNA probes, 1 \( \mu \)g of poly-dIdC, 4 \( \mu \)l of 5 \( \times \) binding buffer (10 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA and 10 mM Tris, pH 7.4), and different concentrations of CadC-his protein. The reaction mixture was then incubated at 25°C for 30 min. Subsequently, 2 \( \mu \)l of 10 \( \times \) EMSA/Gel-shift loading buffer was added to each sample, and the samples were resolved using native PAGE (6% gel) with 0.5 \( \times \) TBE buffer for 2 hr at 100 V. The gel was scanned by a Typhoon FLA 9500 (GE Healthcare).
2.7 | Motility analysis

The WT strain was cultured and exposed to sub-lethal acidic pH as described above in RNA-sequencing analysis. Two microlitres of the diluted culture (OD_{600} = 1.0), alongside the unexposed control, was spotted on LB plates containing 0.3% agar and incubated at 37°C for 12 hr to investigate the swimming capacity. The experiments were repeated three times.

2.8 | Cytotoxicity analysis

The WT strain was cultured and exposed to sub-lethal acidic pH as described above in RNA-sequencing analysis. The culture, along with the unexposed control, was centrifuged with 4,000 g and washed twice by Dulbecco’s modified Eagle’s medium (DMEM). The pellet was resuspended in DMEM containing 10% foetal bovine serum (FBS). The bacteria were diluted 1:10^6, and each bacterial suspension was inoculated into a well of a 24-well plate containing 5 × 10^5 HeLa cells/well to achieve a multiplicity of infection (MOI) of 100. Following infection, the lactate dehydrogenase (LDH) activity of the supernatants was measured at the indicated times using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer’s protocol.

3 | RESULTS

3.1 | Acid tolerance of Vibrio parahaemolyticus

The ATR of V. parahaemolyticus RIMD2210633 was determined by using the inorganic acid HCl and the organic acid citric acid. The WT strain did not survive after an incubation period of 1 hr at pH 4.0, which is lethally acidic for the bacteria unless preincubation in the LB medium with pH 5.5 for 1 hr (Figure 1a). Overall, 16.65% and 1.80% of the bacteria preincubated in sub-lethal acidic (HCl, pH 5.5) could survive with the lethal acidic challenge (HCl, pH 4.0) of 0.5 hr and 1 hr, respectively (Figure 1b). A similar result was also found when citric acid was used (Figure 1c). These results indicate that the adaptation to sub-lethal acidic pH enhanced the survival of V. parahaemolyticus at the lethal acidic pH invoked both by an inorganic acid (HCl) and organic acid (citric acid).

3.2 | The genes responsible for the ATR in Vibrio parahaemolyticus

In this study, we compared the transcriptomes of V. parahaemolyticus exposed to sub-lethal acidic pH conditions with those of unexposed bacteria using RNA-sequencing (RNA-seq) to gain further insight into the genes responsible for the ATR (Table S1). The results showed that 321 genes showed significantly different expression levels, with 234 genes upregulated and 87 downregulated (Figure 2a). Furthermore, a volcano plot of the RNA-seq data was generated to visualize the differentially expressed genes at the sub-lethal acidic pH and assess the quality and comparability of the hybridizations (Figure 2b).

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, the differentially expressed genes were divided into organismal systems (n = 11), metabolism (n = 65), human diseases (n = 14), environmental information processing (n = 43) and cellular processes (n = 33; Figure 2c). Two heat-shock protein genes (VP0651 and VP0821), VPA0586/VPA0287 (groES/groEL), outer membrane protein genes (ompU and ompV) and Na⁺/H⁺ antiporter protein (VP1229) were significantly upregulated under sub-lethal
It was also established that the DNA-binding transcriptional activator (cadC), lysine decarboxylase (cadA) and lysine/cadaverine antiporter (cadB) genes were upregulated in lethal acidic conditions. In addition, virulence-associated genes, including phosphotransferase system (PTS), flagellar assembly and bacterial chemotaxis, were significantly upregulated under the sub-lethal pH conditions. In addition, virulence-associated genes, including phosphotransferase system (PTS), flagellar assembly and bacterial chemotaxis, were significantly upregulated under the sub-lethal
acidic conditions, while the type III secretion system and the two-component system CpxRA (VPA0148/VPA0149) was downregulated. It was speculated that the ATR could influence bacterial pathogenesis in addition to enhancing survival.

3.3 | CadBA modulates the ATR in *Vibrio parahaemolyticus*

RNA-seq analysis indicated that the *cadBA* genes were upregulated when the cells were exposed to the sub-lethal acidic pH (5.5) (Figure 3a), and qRT-PCR confirmed this result (Figure 3b). The location of *cadBA*-cadC genes and their predicted promoter are shown in Figure 3c. The RT-PCR product of the expected size was obtained with the primers spanning the regions from *cadA* to *cadB*. No product was obtained using RNA as the template, and a product of the same size was observed when the genomic DNA was used as the template (Figure 3c), indicating that the *cadB* and *cadA* genes are co-transcribed.

The deletion mutant strains of Δ*cadB* and Δ*cadA* were constructed and assessed to better understand the function of *cadBA* in the ATR. The results showed that the WT, Δ*cadB* and Δ*cadA* strains could not survive in lethal acidic conditions without adaptation to the sub-lethal acidic conditions. When the strains were incubated at the sub-lethal acidic pH for 1 hr, the WT strain could survive at the lethal acidic pH; however, the Δ*cadB* and Δ*cadA* strains could not (Figure 3d), demonstrating that the CadBA protein is necessary for the survival of *V. parahaemolyticus* under the extremely acidic conditions.

3.4 | CadC directly regulates the expression of *cadBA* to mediate ATR

RNA-seq and qRT-PCR indicated that the *cadC* mRNA level was increased when cells were exposed to sub-lethal acidic pH (Figure 3a,b). The transcript level of *cadB* and *cadA* genes was decreased in the Δ*cadC* strain compared with the levels in WT, and the complementary strain *cadC*+ restored the levels to the WT (Figure 4a), indicating that the CadC protein regulates the expression of *cadBA* in *V. parahaemolyticus*. EMSA determined the CadC protein directly bound to the promoter region of *cadBA*, and poly(dI-dC) was added as a nonspecific competitor. As expected, the CadC protein could directly bind to the promoter of *cadBA* in a concentration-dependent manner (Figure 4b). Additionally, the gyrB promoter was employed as a negative control, and the CadC

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**FIGURE 3** CadBA is responsible for the ATR in *Vibrio parahaemolyticus*. (a) The *cadBA* and *cadC* mRNA levels based on the RNA-seq. (b) qRT-PCR analysis of the *cadBA* and *cadC* mRNA levels under the sub-lethal acidic condition. The results are shown normalized to the gyrB mRNA levels and were determined by the ΔΔCt method. The differences are shown relative to the levels at pH 7.5. *p < .05, ***p < .001, t test. (c) The genetic environment of the *cadBA*-cadC genes in *V. parahaemolyticus* chromosome. Arrows indicate the predicted promoter regions and RT-PCR was used to determine whether *cadA* and *cadB* are co-transcribed. (d) The survival of *V. parahaemolyticus*. The WT, Δ*cadA*, and Δ*cadB* strains were exposed to the lethal acidic pH (4.0, HCl) for 1 hr with or without preadaptation at the sub-lethal acidic pH (5.5, HCl) for 1 hr. The bacteria were then serially diluted (10-fold) and spotted onto LB agar plates [Colour figure can be viewed at wileyonlinelibrary.com]
CadC directly regulates the expression of cadBA to modulate the ATR in *Vibrio parahaemolyticus*. (a) qRT-PCR analysis of the cadBA and cadC mRNA levels in the WT, ΔcadC and cadC+ strains. Total RNA was isolated from the WT, ΔcadC and cadC+ strains cultured in the LB medium at pH 7.5 for 5 hr. The results are shown normalized to the gyrB mRNA levels and were determined by the ΔΔCT method. The differences are shown relative to the levels in the WT. *p < .05, t test. (b) EMSA assays were performed with the CadC protein and cadBA promoter. The CadC protein at the indicated concentration and 20 ng of FAM-labelled P_cadBA DNA probe was used. The shifts were verified by a nonspecific competitor DNA [poly(dI:dC)], and the gyrB promoter was used as a negative control. (c) The survival of *V. parahaemolyticus*. The WT, ΔcadC and cadC+ strains were exposed to the lethal acidic pH (4.0, HCl) for 1 hr with or without preadaptation at the sub-lethal acidic pH (5.5, HCl) for 1 hr. The bacteria were then serially diluted (10-fold) and spotted onto LB agar plates [Colour figure can be viewed at wileyonlinelibrary.com]

The regulon of CadC protein assessed using RNA-seq. (a) Pie charts represent differentially transcribed genes between the WT and ΔcadC strains cultured in LB at pH 7.5. (b) A volcano plot was generated to visualize the differentially expressed genes in ΔcadC. The x-axis represents the log2 of the fold change plotted against the −log_{10} of the adjusted false discovery rate. Red and blue points indicate the up- and downregulated genes, respectively. (c) Venn diagrams showing the genes whose expression is co-regulated by sub-lethal acidic pH (5.5, HCl) and CadC protein [Colour figure can be viewed at wileyonlinelibrary.com]
protein at the highest concentration, could not bind to the negative control DNA (Figure 4b).

The ATR of ΔcadC strain was also investigated. Results showed that the WT, ΔcadC, and cadC+ strains could not survive at the lethal acidic pH (4.0) without adaptation. In contrast, the WT and cadC+ strains could survive at the lethal acidic pH after adaptation to the sub-lethal acidic pH (5.5) for 1 hr. The ΔcadC strain could not survive at the lethal acidic pH after pre-adaptation (Figure 4c), indicating that the CadC protein is involved in regulating the ATR. These results suggest that CadC protein could directly bind to the promoter region of cadBA and regulate the expression of cadBA to modulate the ATR in V. parahaemolyticus.

3.5 | CadC-dependent and CadC-independent genes responsible for the ATR in Vibrio parahaemolyticus

In the sub-lethal acid (pH 5.5, HCl), 321 genes were differentially expressed by the RNA-seq (Figure 2a). CadC protein as a transcription factor could directly regulate the expression of cadBA to modulate the ATR. RNA-seq was used to identify the genes regulated by the CadC protein to determine the CadC-dependent and CadC-independent genes responsible for the ATR. The transcriptomes of ΔcadC and WT were compared to identify the changes in gene expression patterns in the absence of a functional CadC protein (Table S2). Results showed that 565 genes were expressed differently in ΔcadC; 292 genes were upregulated, while 273 genes were downregulated (Figure 5a). The volcano plot of the RNA-seq data was generated to visualize the differentially expressed genes of the ΔcadC strain and to assess the quality and comparability of the hybridizations (Figure 5b).

The RNA-seq data of the sub-lethal acid and ΔcadC were compared, 113 genes were identified as co-regulated (Figure 5c and Table S3). These genes could be considered responsible for the adaptation to the sub-lethal acidic pH through CadC (113 genes) and independently of CadC (208 genes). Among the CadC-dependent genes, a Na+/H+ antiporter protein (VP1229) was upregulated at the sub-lethal acidic pH and downregulated in the ΔcadC strain; the CadC protein may regulate the expression of VP1229 to modulate the ATR. Furthermore, cadB and cadA genes were downregulated
by CadC and induced at the sub-lethal acidic pH; these results were confirmed using qRT-PCR (Figure 3b and Figure 4a). CadC regulates the expression of formate dehydrogenase, D-lactate dehydrogenase, glyceral-3-phosphate dehydrogenase, and 6 PTS proteins. The two heat-shock protein genes (VP0651 and VP0821) and groES/groEL (VPA0586/VPA0287) were downregulated in the ΔcadC strain. These data suggested that adaptation to sub-lethal acidic pH increases the expression of the genes that modulate the ATR through CadC-dependent or CadC-independent way in *V. parahaemolyticus*.

3.6 | Motility and cytotoxicity were induced in sub-lethal pH conditions

Motility and cytotoxicity were identified as the main virulence factors of *V. parahaemolyticus* (Gu et al., 2019; Zhang & Orth, 2013). RNA-seq data showed that polar flagellar genes were upregulated under sub-lethal acidic conditions (Figure 6a). The swimming motility and cytotoxicity of *V. parahaemolyticus* were assessed after the adaptation to a sub-lethal acidic pH (5.5) for 1 hr. The results showed that the swimming motility of WT was significantly increased post-adaptation to the sub-lethal acids, including the inorganic acid (HCl) and organic acid (citric acid) (Figure 6b,c). The cytotoxicity of WT strain on HeLa cells was also significantly increased after the cells adapted to the sub-lethal acidic pH (Figure 6d). These results indicated that adaptation to sub-lethal acidic conditions could increase the virulence of *V. parahaemolyticus*.

4 | DISCUSSION

*Vibrio parahaemolyticus* were highly prevalent in shrimp, fish and seafood products (Lee et al., 2019; Lei et al., 2020; Li et al., 2020; Tan et al., 2020). A previous study indicated that *V. parahaemolyticus* was detected in 3.6% of natural feed samples and 83.3% of fresh feed samples, respectively (Yingkajorn et al., 2014). The feed acidifiers have been used to increase the growth of aquatic animals. Our study identified the CadC-CadBA gene cluster in the *V. parahaemolyticus* under environmental stress conditions in further studies. Previous study revealed that the supplementation of organic acids in diet could improve the feed conversion ratio and the survival rate of Oreochromis niloticus and red hybrid tilapia (Asriqah et al., 2018). Fulvic acids in feed supplement could also improve the growth performance and intestinal health of rainbow trout, loach and crayfish (Gao et al., 2017; Lieke et al., 2021; Zhang, 2018). The application of citric acid in the feed supplement could mitigate the distal intestine of juvenile turbot, while formic acid could increase the bioavailability of minerals in rainbow trout (Vielma & Lall, 1997; Zhao et al., 2019). Our results indicated that the sub-lethal acidic conditions could increase the survival ability and virulence of *V. parahaemolyticus*, in which the CadC-CadBA plays an essential role in the regulation of ATR. This phenomenon is reported in the other pathogens including *Salmonella* and *V. cholerae* (Lee et al., 2008; Merrell & Camilli, 1999). These results indicated that the supplementation of acidifiers in the feed may increase the virulence and survival of *V. parahaemolyticus* in the aquaculture.

The CadC-CadBA system was confirmed to be involved in the ATR, which could protect bacteria against acidic conditions. This study identified the CadC-CadBA gene cluster in the *V. parahaemolyticus* and further identified the expression difference of ATR genes in CadC-dependent or CadC-independent ways by RNA-seq. Under sub-lethal acidic conditions, 321 genes were significantly differentially expressed, including the heat-shock protein, outer membrane protein, metabolism, two-component system and virulence (Figure 2). Two genes encoding heat-shock proteins (VP0651 and VP0821) were significantly upregulated under sub-lethal acidic conditions, which was agreed to a previous study revealed that adaptation to sub-lethal acidic conditions could enhance the survival of *V. parahaemolyticus* at high temperatures (Chiang et al., 2014). In this study, the heat-shock-related genes VPA0586/VPA0287 (GroES/GroEL) were significantly upregulated when the cells were adapted to the sub-lethal acidic pH, which is consistent with a previous study result in *Campylobacter jejuni* (Varsaki et al., 2015). The VPA0586/VPA0287 genes were responded to acidic stress in a CadC-dependent manner and significantly downregulated in the
ΔcadC strain (Table S3). Two outer membrane protein genes (ompU and ompV) were upregulated under sub-lethal acidic conditions, which was known to involve in the response to environmental stress (Kao et al., 2009; Merrell et al., 2001; Wu et al., 2006). Twenty-one genes related to the two-component system were significantly differentially expressed under sub-lethal acidic conditions including the CpxRA, which was known to involve in the growth of E. coli under acidic pH (Xu et al., 2020). The genes responsible for the ATR were expressed in a CadC-independent manner.

The virulence-associated genes involved in the bacterial secretion system, flagellar assembly and bacterial chemotaxis were significantly differentially expressed under sub-lethal acidic conditions (Figure 2). Twenty-four flagellar assembly genes and nine bacterial chemotaxis genes were upregulated under sub-lethal acidic conditions. Previous studies showed that low motility was associated with acid stress in E. coli and S. Typhimurium (Ryan et al., 2015; Soutourina et al., 2002), whereas another study indicated that the expression of flagellar genes was induced at low pH in E. coli, which was consistent with our results by RNA-seq (Maurer et al., 2005). The cytotoxicity of pathogens was reported to be induced after adapting to sub-lethal acidic conditions (Yeung & Boor, 2004). Our study demonstrated that the motility and cytotoxicity of V. parahaemolyticus were significantly increased after adapting to sub-lethal acidic pH (Figure 6). Further studies are required to investigate the mechanism of how low pH conditions could influence the regulation of motility in V. parahaemolyticus.

This study confirmed that CadC and CadBA could modulate the ATR system in V. parahaemolyticus. The CadC protein could directly bind to the promoter of cadBA and regulate the expression of CadBA (Figure 7). Additionally, RNA-seq data showed that 113 genes were involved in ATR in a CadC-dependent way, whereas 208 genes modulated the ATR independently to CadC. Motility and cytotoxicity analysis indicated that adaptation to a sub-lethal acidic pH could increase the virulence of V. parahaemolyticus (Figure 7). This study demonstrated that the adaptation of V. parahaemolyticus under the sub-lethal acidic conditions could increase its virulence and survival ability, which increase the potential risk of disease outbreaks in aquaculture.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data sets supporting the results of this article are included within the article and its additional files. The RNA-Seq data in fastq format have been deposited to the European Nucleotide Archive with the accession number PRJEB38562.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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