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Impact of Simulated pH Conditions on Phenotypic Expression in Shrimp Pathogenic and Non-Pathogenic *Vibrio campbellii* Strains

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Abstract

Environmental pH fluctuation in oceanic and marine ecosystems can significantly impact the distribution and behavior of pathogenic *Vibrio* species, including their interactions with marine invertebrates such as crustaceans. This study focused on *Vibrio campbellii*, a common shrimp pathogen, and its phenotypic responses to varying pH conditions. Both pathogenic strain HY01 and non-pathogenic strain ATCC BAA-1116 were cultured in 30 pL/L Luria-Bertani Sea Salt under 3 pH conditions, including pH 6 (slightly acidic), pH 8 (representing the oceanic pH), and pH 9 (alkaline). Growth patterns and phenotypic traits were evaluated. Results revealed no significant growth difference between the 2 strains under the different pH conditions, although the non-pathogenic strain showed a slight growth reduction at pH 9 during the exponential phase. Both strains were able to buffer environmental pH shifts, adjusting to near-oceanic pH levels (around pH 8). At pH 9, a stressor level for *V. campbellii*, delays were observed in bioluminescence, biofilm formation, exopolysaccharide production, shrimp surface colonization, motility, and caseinase production, affecting both strains. In contrast, mildly acidic conditions (pH 6) induced the highest expression of several phenotype traits. Statistical analyses indicated significant interactions between strain type and pH levels in influencing phenotypic expression. In conclusion, the pathogenic *V. campbellii* strain HY01 exhibited greater adaptability and virulence across various pH conditions compared to the non-pathogenic ATCC BAA-1116, emphasizing pH as a critical environmental factor in shaping the growth and pathogenic potential of *V. campbellii*. Our studies provide valuable insights into managing pH conditions in aquaculture environments to optimize proper shrimp cultivation and prevent cross-contamination of *V. campbellii* from seawater habitats to farms. These findings provide a physiological profile of *Vibrio* under pH stress, which can support the development of predictive outbreak models to assess the risk of luminous vibriosis, especially in to seasonal changes and ocean acidification.

Keywords: Aquaculture, Aquaculture management, Biofilm formation, Climate change, Luminous vibriosis, Ocean acidification, pH fluctuation, Shrimp pathogen, *Vibrio campbellii*

Introduction

Environmental factors play a crucial role in shaping the distribution, prevalence, and virulence of pathogenic *Vibrio* species. In recent decades, increasing climate variability has led to significant ecological changes that affect microbe-host interactions [1]. Among these changes, seawater pH is known to fluctuate diurnally, and global ocean pH levels are projected to become increasingly acidic over the coming decades due to factors such as rising atmospheric CO_2 [2]. *Vibrio* spp. are naturally free-living in marine environments, including oceans, estuaries, and coastal regions, where the pH typically ranges around 8 (slightly alkaline). These bacteria can transition between different environments and enter aquaculture systems, leading to contamination of marine animals. While aquaculture systems are generally managed to maintain water pH within an optimal mildly alkaline range (pH 7.5 - 8.5), fluctuations are common due to various factors, including the types of

animal cultured, waste management practices, cultivation systems, and seasonal changes [3,4]. As a result, pH variation may be an important factor influencing the survival and virulence of pathogenic *Vibrio* species.

Halophilic *Vibrio* species are known for their ability to grow across a board range of pH levels, which reflects their ecological origins and their adaptability to colonize both human and animal hosts. Previous studies have shown that pathogenic strains, such as *V. vulnificus* and *V. parahaemolyticus*, can grow across a wide pH range, from pH 5 to 9.5 [5]. Many *Vibrio* species are capable of tolerating both acidic conditions (pH < 5) and alkaline conditions (pH > 8). Notably, human pathogens like *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* can thrive in fluctuating environmental pH, transitioning from their natural alkaline marine habitats to the acidic conditions found in the human stomach [6-9]. In marine *V. fischeri*, pH act as external inducer, ranging from 5.0 to 10.6, to change bacterial lifestyle from free-living to colonization on its mutualistic animal host *Euprymna scolopes* [10]. The ability to adapt to a broad pH spectrum demonstrates the resilience of *Vibrio* spp. to both acidic and alkaline conditions and their capacity to survive transitions between these extremes.

V. campbellii is commonly found on the surfaces and in the guts of marine crustaceans, as well as in free-living populations in seawater and other marine environments. It can spread from these natural habitats to aquaculture systems, where it may cause cross-contamination and infections in farmed animals. In particular, *V. campbellii* is a known pathogen of marine shrimp, where it causes luminous vibriosis, a condition that leads to significant shrimp mortality [11,12]. In previous study by Rattanama *et al.* [13], the pathogenic *V. campbellii* strain HY01, isolated from shrimp mortalities caused by luminous vibriosis in Thailand, exhibiting high virulence, causing significant mortality in black tiger shrimp within 12 h post-injection. In contrast, the non-pathogenic strain ATCC BAA-1116, isolated from the ocean environment, did not induce mortality in shrimp. However, *V. campbellii* HY01 and ATCC BAA-1116 was once misidentified as the close relative of *V. harveyi* based on genotypic and phenotypic similarities [14]. Notably, self-bioluminescence is dominant feature emitted by both *V. campbellii* and *V. harveyi*, which has previously led to misinterpretation of luminous vibriosis observed through shrimp body fluorescence. However, molecular and bioinformatic techniques have been developed to improve the accuracy and precision in distinguishing between *V. harveyi* and *V. campbellii* [15,16]. A recent study by Kumar *et. al* [12] compared the phenotypic and genotypic traits of pathogenic *V. campbellii* strains, identifying those that are highly virulent and responsible for luminous vibriosis in Indian shrimp hatcheries. Despite these findings, further research into the phenotypic features of *V. campbellii* related to virulence and adaptation is necessary. Such studies will help distinguish between pathogenic and non-pathogenic strains and provide a deeper understanding of their responses to fluctuating environmental factors, particularly pH conditions. The non-pathogenic *V. campbellii* ATCC BAA-1116 was shown to exhibit an acidic-responsive system activated at pH levels ranging from 5.4 to 6.0, indicating its adaptive capacity under low pH stress [17]. Moreover, previous studies have demonstrated that the shrimp pathogenic *V. campbellii* strain HY01 can grow within a pH range of 5.5 to 8.6, similar to other *Vibrio* species [18]. Additionally, strain HY01 showed strong resistance to acidic conditions at pH 5, a factor that enhanced its hemolytic activity and virulence in the insect model *Galleria mellonella* [18]. This finding underscores the importance of pH as a critical factor in the virulence of pathogenic *V. campbellii* in animal model. In addition, environmental pH also affects the physiological changes in the animal host, leading to facilitate bacterial infection and colonization. Several studies have reported that decreased pH, resulting in more acidic condition, can suppress growth, survival, immune function, and resistance to bacterial pathogens in shrimp [19,20]. Therefore, fluctuations in seawater pH are not only important for the survival and quality of shrimp but also influence the distribution and pathogenicity of *V. campbellii*. Despite numerous studies reporting the survival of *Vibrio* spp. under various pH levels, effects on pH-related phenotypic expression of *V. campbellii* have been under-explored. In this study, we aimed to investigate and compare growth and phenotypic responses, including bioluminescence, biofilm formation, exopolysaccharide, surface adhesion, swimming motility, and protease production, of the shrimp pathogenic *V. campbellii* strain HY01 and the non-pathogenic strain ATCC BAA-1116 to simulated pH conditions.

Materials and methods

Bacterial strains and growth condition

In this study, we compared the adaptability of 2 *V. campbellii* strains: The shrimp pathogenic strain HY01 [13] and the non-pathogenic strain ATCC BAA-1116 [21], under simulated pH conditions. Oceanic pH levels currently average around 8.0 to 8.2, but computational models predict a gradual decrease in pH, which values potentially reaching approximately 7.0 by 2100 [22]. In shrimp aquaculture, water pH is typically maintained between 7.5 and 8.5 [3]; though fluctuations between approximately pH 7.94 to 9.40 can occur due to factors such as time of day or seasonal changes [23,24]. To mimic these fluctuations, 3 pH values were selected to test the survival capabilities of both pathogenic and non-pathogenic *V. campbellii* strains: pH 6 (strong acidification), pH 8 (representing typical oceanic conditions), and pH 9 (extreme alkalinity, associated with phenomena such as algae blooms and urea accumulation in shrimp pond).

Luria-Bertani broth (LB) (Difco™, USA), containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride, was supplemented with 30 pL/L artificial sea salt (Marinium Reef Sea Salt, Thailand) to prepare Luria-Bertani Sea Salt (LBS). The pH of LBS was monitored using a pH meter (OHAUS, China). Initially, the pH of LBS was measured before being adjusted to desired levels. For the preparation of 400 mL LBS, the initial pH of LBS was approximately 6.77. To adjust the pH, 0.3 mL of 5 M HCl was added to achieve pH 6, and 1 and 2 mL of 5 M NaOH were added to reach pH 8 and 9, respectively. The prepared LBS was sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C until use. Two *V. campbellii* strains were pre-cultured in LBS at normal pH (~ 6.5) and incubated at 30 °C with shaking at 150 rpm for 4 - 5 h to reach the exponential growth phase. The bacterial cultures in exponential phase were adjusted to 0.5 McFarland turbidity standard (approximately 1.0×10^8 CFU/mL) and then diluted to a final centration of 1.0×10^5 CFU/mL for testing the effects of pH on growth and phenotypic traits.

Growth and bioluminescence kinetic assays

The growth and bioluminescence of both *V. campbellii* strains were monitored in a 96-well microplates. For growth detection, 200 µL of 30 pL/L LBS adjusted to pH 6, 8, or 9 was inoculated with each strain and placed in a clear microplate. For bioluminescence detection, a separate 96-well white microplate was used, and the luminescence was measured. Plates were incubated at 30 °C with orbital shaking at 100 rpm, and optical density (OD) at 600 nm to measure bacterial growth based on cell turbidity and at 490 nm to detect emitted bluish-green light were measured over a 24 h-period using a microplate reader (LUMIstar, BMGLABTECH, Germany). Growth rates, generation times, and total luminescence (relative light units; RLU) were then calculated for each strain under the different pH conditions.

Measurement of pH variability

V. campbellii strains were cultured in Erlenmeyer flasks containing 100 mL of 30 pL/L LBS adjusted to pH 6, 8, or 9, and incubated at 30 °C with shaking at 150 rpm. The pH was measured at 0, 3, 6, 9, 12, and 24 h using a pH meter. Viable cell counts were also determined at these time points to monitor bacterial growth under the varying pH conditions.

Biofilm biomass determination

Biofilm formation by both *V. campbellii* strains was assessed using the crystal violet (CV) assay [25]. Strains were cultured in 96-well plates with 200 µL of 30 pL/L LBS at pH 6, 8, or 9 and incubated at 30 °C for 48 without shaking to allow biofilm formation. After incubation, wells were washed 3 times with sterilized distilled water (DW) to remove non-adherent cells. The biofilms were then stained with 200 µL of 0.1 % CV for 30 min, followed by washing with DW to remove excess dye. The bound CV was solubilized with 95 % ethanol, and the absorbance at 570 nm was measured using a microplate reader to quantify biofilm biomass.

Indirect exopolysaccharide (EPS) production assay

EPS production by both *V. campbellii* strains was visualized Confocal Laser Scanning Microscopic (CLSM). Biofilms were allowed to form on sterilized glass slides [26,27] in 24-well plates containing 30 pL/L LBS adjusted to pH 6, 8 and 9 and incubated at 30 °C for 48 h without shaking. After incubation, free-living bacteria was removed, and the glass slides were gently rinsed with sterilized DW to eliminate non-attached cells. The biofilms were fixed with 2.5 % glutaraldehyde

(Sigma, USA), washed with DW, and stained with FITC-conjugated concanavalin A (FITC-ConA) using FluoroTag^{TM} conjugation kit (Sigma-Aldrich, USA) and propidium iodide (PI; Sigma, USA) to visualize the EPS. Fluorescence was detected using CLSM (LSM800 ZEISS, Germany) at excitation/emission wavelengths of 495/519 nm for FITC-ConA and 548/561 nm for PI. The CLSM images were captured in Z-stack mode using Zen v. 2.3 software (ZEISS, Germany) with a 20× objective lens (numerical aperture 0.4). The resolution was approximately 0.754 µm for FITC-ConA and 0.834 µm for PI.

Adhesion to shrimp shell surfaces

The preparation of shrimp shells followed the protocol by Han *et al.* [28]. Dead *Litopenaeus vannamei* shrimp were purchased from a local seafood market in Hat Yai, Songkhla, Thailand. The shells were removed from shrimp meat, thoroughly washed to remove residual organic matter, and then cut into 1×1 cm^2 pieces. The shells were decontaminated by immersion in formaldehyde for at least 1 h. The shell pieces were placed in 24-well plates containing 30 pL/L LBS at pH 6, 8, or 9. Bacterial suspensions of strains HY01 and ATCC BAA-1116 were added to the wells and incubated overnight at 30 °C without shaking. After incubation, non-adhered cells were removed, and the shells were gently washed with DW. Biofilms on the shrimp shells were fixed in 2.5 % glutaraldehyde, dehydrated through a series of ethanol concentrations (30, 50, 60, 70, 80, 95, and 100 % (v/v)), and coated with gold. The biofilm structures were then observed using scanning electron microscopy (SEM) (Hitachi SU3900, Japan). The SEM images were captured at an accelerating voltage of 20.0 kV, a resolution of 2 nm, a working distance of 10.0 mm, and a magnification of ×2,000.

Swimming motility assay

Swimming motility was assessed as described by Santhakumari *et al.* [29], with minor modifications. The 2 *V. campbellii* strains were inoculated in the exponential growth phase onto semi-solid 0.3 % (w/v) LBS agar plates adjusted to pH 6, 8, and 9, and incubated at 30 °C for 16 h. The diameter of the motility zones was measured using a Vernier caliper.

Caseinase assay

Caseinase activity was determined using the method of Han *et al.* [30]. LBS agar plates were supplemented with 2 % (w/v) skim milk powder and adjusted to pH 6, 8, and 9. The bacterial strains were inoculated onto the plates and incubated at 30 °C for 72 h. The diameters of the colonies and clear proteolytic zones were measured using a Vernier caliper.

Statistical analysis

All experiments were performed in biological triplicates, and data are presented as means ± standard deviations. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS, version 20.0). Levene's test was used to assess the homogeneity of variances, and the normality of data distributions was evaluated using the Shapiro-Wilk test. Since both tests indicated equal variances and normal distributions, a 2-way ANOVA was used to analyze the interactions between pH and bacterial strains on the dependent variables (phenotypic tests). Multiple comparisons between groups (n = 6) were performed using Tukey's post-hoc test.

Results and discussion

Growth kinetics of pathogenic and non-pathogenic *V. campbellii* strains are altered by pH levels

To determine bacterial growth trends influenced by acidic and alkaline conditions, the growth kinetics of the shrimp pathogenic *V. campbellii* strain HY01 and the non-pathogenic strain ATCC BAA-1116 were assessed under simulated conditions at pH 6, 8, and 9 for 24 h. Both strains remained viable across the entire pH range of 6 to 9 (**Figure 1(A)**). Notably, the pathogenic strain HY01 entered the exponential phase more rapidly under all pH conditions compared to the non-pathogenic strain. At 24-h of incubation, the highest cell turbidities (OD 600 nm) for both strains were observed at pH 6. For strain HY01, there was no significant difference in cell proliferation or generation time across the pH conditions (**Figures 1(B)** and **1(C)**); However, cell densities in the stationary phase were slightly lower at pH 8 and 9 compared to pH 6. In contrast, at pH 9, strain ATCC BAA-1116 exhibited delayed cell proliferation during the early growth stages, reaching the exponential phase after 7.5 h of incubation (**Figure 1(A)**). The lowest growth rate

and longest doubling time were observed for the non-pathogenic strain at pH 9 (**Figures 1(B)** and **1(C)**). These findings are consistent with those reported by Prayitno and Latchford [31], who showed that *V. harveyi*, the species genetically closest to *V. campbellii*, is capable of growing across a broad pH range (from pH 5.5 to 9). Similarly, Gundogdu *et al.* [32] demonstrated the combined effects of pH and temperature on the growth and cell morphology of *V. harveyi*. Statistical analysis of the growth data revealed significant effects of both pH and strain on generation time. While both factors individually influenced growth rate, there was no significant interaction between pH and strain (pH×strain) (**Table 1**). This indicates that the effect of pH on *V. campbellii* growth was consistent across both strains. Notably, the pathogenic strain HY01 exhibited significantly greater survivability than the non-pathogenic strain ATCC BAA-1116 under all tested pH conditions, as confirmed by multiple comparison analysis.

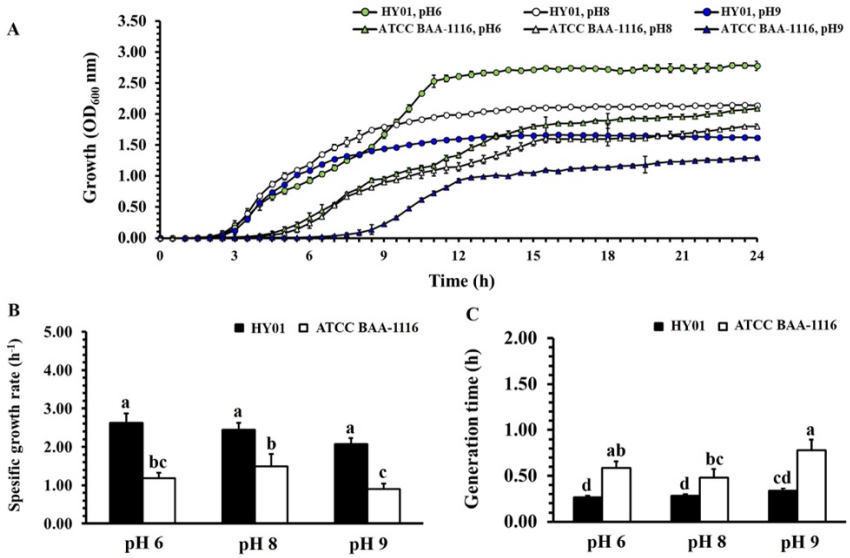

Figure 1 The effect of acidic and alkaline pH conditions on growth of shrimp pathogenic *V. campbellii* strain HY01 and non-pathogenic *V. campbellii* strain ATCC BAA-1116 (A). The specific growth rate (B) and generation time (C) of both strains were also calculated based on mid-exponential phase of growth curve (A). Levene's test and Shapiro-Wilk test indicated equal variances and normal distributions (p-value ≥ 0.05). The lowercase letters represent the statistical analysis using Tukey's post hoc test for comparison among groups at p-value < 0.05. Different lowercases denote statistically significant differences, while the same lowercase letters indicate no statistically significant differences.

Table 1 Two-way ANOVA results showing the interaction effects of pH conditions and bacterial strains on growth and phenotypic characteristics of *V. campbellii*.

Factors	*V. campbellii* strains		pH		*V. campbellii* strains×pH	
	***F*-value**	***P*-value**	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Growth rate	151.237	< 0.001*	9.766	0.003*	2.034	0.174
Generation time	96.422	< 0.001*	10.318	0.002*	4.714	0.031*
Bioluminescence	4.834	0.048*	9.901	0.003*	4.104	0.044*
Biofilm formation	6.329	0.027*	47.929	< 0.001*	5.435	0.021*
Swimming motility	40.808	< 0.001*	39.238	< 0.001*	0.826	0.461
Protease production	18.323	0.001*	19.693	< 0.001*	0.209	0.814

Note: "*" indicates a statistically significant interaction at $P < 0.05$.

The alteration of pH across *V. campbellii* growth stages

Both *V. campbellii* strains were cultured on a large-scale in LBS at varying initial pH conditions, and the pH levels were monitored throughout the bacterial growth phases. The results demonstrated that initial pH values ranging from 6 to 9 did not significantly suppress the population of the pathogenic strain HY01. Across all conditions, the pH levels gradually decreased by 6 h of incubation, followed by an increase, eventually stabilizing at approximately pH 8 after 24 h of incubation (**Figure 2(A)**).

In contrast, the non-pathogenic strain ATCC BAA-1116 exhibited optimal growth at pH 6 and 8. However, at pH 9, the strain showed slower growth during the 1st 12 h of incubation, although the final cell concentration at 24 h did not differ significantly from that observed under other conditions (**Figure 2(B)**). These observations suggest that *V. campbellii* strains are capable of adapting and modifying their surrounding environment to achieve an optimal pH for growth. Despite these findings, the mechanisms underlying *V. campbellii*'s ability to thrive under pH stress remain incompletely understood. Recent work by Schwarz *et al.* [17] provides insight into this adaptation, identifying the role of AphB, a transcriptional regulator, and the cad operon, a pH-responsive system, in *V. campbellii* ATCC BAA-1116. At pH levels below 6.6, AphB functions as a pH sensor, activating the CadC regulator of the *cadBA* operon. CadC then stimulates the expression of *cadBA*, where *cadA* encodes lysine decarboxylase, and *cadB* encodes a lysine/cadaverine transporter. This process facilitates the export of lysine or cadaverine, thereby increasing the extracellular pH to a more alkaline level and maintaining pH homeostasis. The previous study clearly demonstrated that a pH shift from 5.0 to above 6.8 in the medium of 24-h cultured *V. campbellii* ATCC BAA-1116 was caused by the accumulation of cadaverine, which was detected using the pH indicator bromocresol purple [17]. On the contrary, mechanisms for responding to alkaline stress are less well-documented in *V. campbellii* and other marine *Vibrio* species. A study by Nozaki *et al.* [33] was the 1st to characterize the role of NhaD, a Na^+/H^+ antiporter in *V. parahaemolyticus*, which is active at pH levels above 8.5 but inactive at neutral pH (7.0 - 7.5). Similarly, in *V. cholerae*, 3 Na^+/H^+ antiporters (Vc-NhaABD) and an NADH-quinone oxidoreductase pump have been identified as critical components of the Na^+ resistance mechanism under extreme alkaline conditions and salt stress [34]. In other bacteria, such as *E. coli*, the NhaAB antiporter regulates pH homeostasis by facilitating proton exchange when Na^+ levels increase under highly alkaline conditions [35]. Although there is no report of high alkaline pH changes in the medium driven by the Na^+/H^+ antiporter system, this mechanism may represent a possible pH adaptation strategy in *V. campbellii*.

In addition, a recent study by Rahmani *et al*. [36] reported that marine *V. tapetis* cultured at pH 6.00 to 8.70 exhibited a shift to approximately pH 7.90 after incubation, with different trends observed depending on the substrates and culture conditions. This finding is supported by the study of Kostiuk *et al.* [37], which indicated that an increase in pH towards more alkaline conditions in *V. cholerae* was associated with the utilization of citrate as a substrate in citrate metabolism. These results suggest that pH adaptation may occur through the utilizing substrates for energy generation, which by-products as amino acids, accumulating outside the cells and causing changes in the surrounding pH [38]. However, our study did not identify the specific amino acids excreted into the culture medium.

External pH fluctuations in *V. campbellii* may result from various factors, possible representing a pH-responsive mechanism that modifies the surrounding cell environment to be more favorable for cellular processes, ensuring sufficient energy for replication. This phenomenon was particularly evident in the growth pattern of ATCC BAA-1116, which exhibited slow growth during the 1st 12 h, followed by a rapid increase in cell concentration, showing no significant differences compared to other pH conditions (**Figure 2(B)**). In contrast, the pathogenic strain HY01 demonstrated faster replication, indicating its superior adaptation to pH ranges from 6 to 9. Nonetheless, our study did not fully elucidate the mechanisms underlying *V. campbellii*'s responses to pH stress. This presents an interesting gap for further exploration, particularly in understanding the bacterial mechanisms related to pH adaption and their link to virulence and pathogenicity through molecular studies.

These previous findings support our observation that *V. campbellii* strains cultured at pH 6 (slightly acidic) and pH 9 (strongly alkaline) adjust their surrounding

environment to a pH of approximately 8 (**Figure 2(A)**). This adaptation aligns with the conditions commonly found in their natural habitats, such as oceanic and shrimp farming environments, which are typically slightly alkaline, and the accumulation of amino acid in culture from energy production.

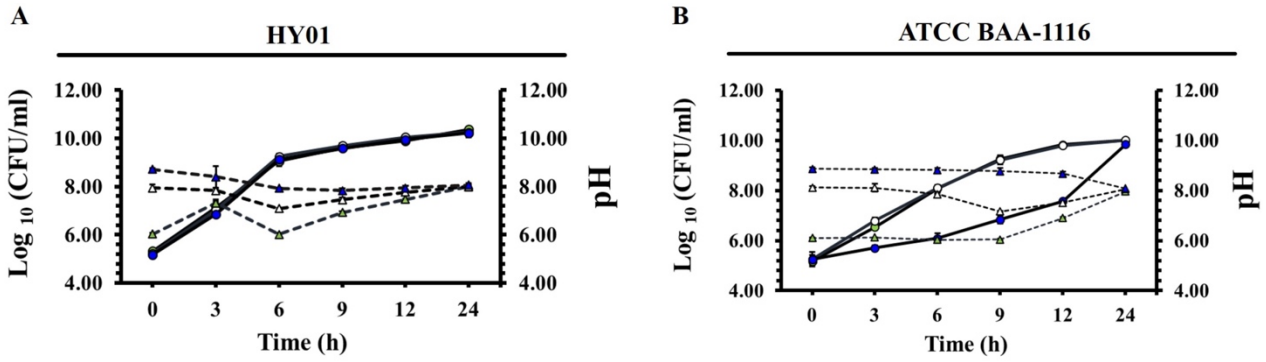

Figure 2 Variation in pH during the growth of shrimp pathogenic *V. campbellii* strain HY01 (A) and non-pathogenic *V. campbellii* strain ATCC BAA-1116 (B). Both strains were grown in 30 pL/L LBS adjusted to pH 6, 8, and 9.

Bioluminescence kinetics of pathogenic and non-pathogenic *V. campbellii* strains are altered by pH levels.

The light emissions of both *V. campbellii* strains were analyzed under varying pH conditions. The pathogenic strain HY01 exhibited the highest luminescence (2.00×10^6 RLU at 9 h of incubation) when grown in LBS at pH 6, which correlated with the highest cell density recorded at that time. In contrast, alkaline pH slightly reduced the total bioluminescence of strain HY01. For the non-pathogenic strain ATCC BAA-1116, no significant differences in total bioluminescence were observed across the tested pH conditions (**Figures 3(A)** - **3(B)**). At pH 9, bioluminescence in both *V. campbellii* strains was delayed, indicating a potential pH-dependent lag in light production (**Figures 3(A)** and **3(C)**). In *V. campbellii*, bioluminescence is primarily regulated by the concentration of signaling molecules that activate the transcriptional regulator LuxR within the quorum sensing system [39]. Several studies have shown that during the early to exponential growth phase, the production of autoinducer-2 (AI-2), a universal molecule produced by many bacterial genera, deceased under neutral to alkaline pH conditions (> 7.0). In contrast, an increase in AI-2 activity was observed at slightly acidic pH (≤ 6.0) [40-42]. This decrease in AI-2 production at alkaline pH may subsequently suppress the function of LuxR, which activates the expression of the *lux* operon and controls bioluminescence. These findings may explain the delay in bioluminescence observed under alkaline conditions. However, luminescence patterns were not directly attributable to the initial pH, as all cultures gradually shifted toward a slightly alkaline pH during incubation. This made it challenging to pinpoint the exact pH at the time of maximum or delayed luminescence. In luminous marine photobacteria, maximum luminescence typically occurs across a neutral to mildly alkaline pH range (pH 7 to 9) and decreases at acidic or highly alkaline pH levels [43]. Similarly, wild-type *V. harveyi* exhibits peak luminescence across pH 6.5 to 8.5, influenced by extracellular Na^+ levels and the ionic balance between the intracellular and extracellular environments [44]. Statistical analysis confirmed that both pH and bacterial strain significantly influenced bioluminescence (**Table 1**). The distinct luminescence patterns of pathogenic and non-pathogenic *V. campbellii* strains were consistent with findings from Calogero *et al.* [45], who reported that bioluminescence in marine luminous bacteria depends on strain-specific characteristics and environmental factors such as pH, temperature, and NaCl concentration. This study highlights external pH as a critical factor affecting bioluminescence in marine *V. campbellii*.

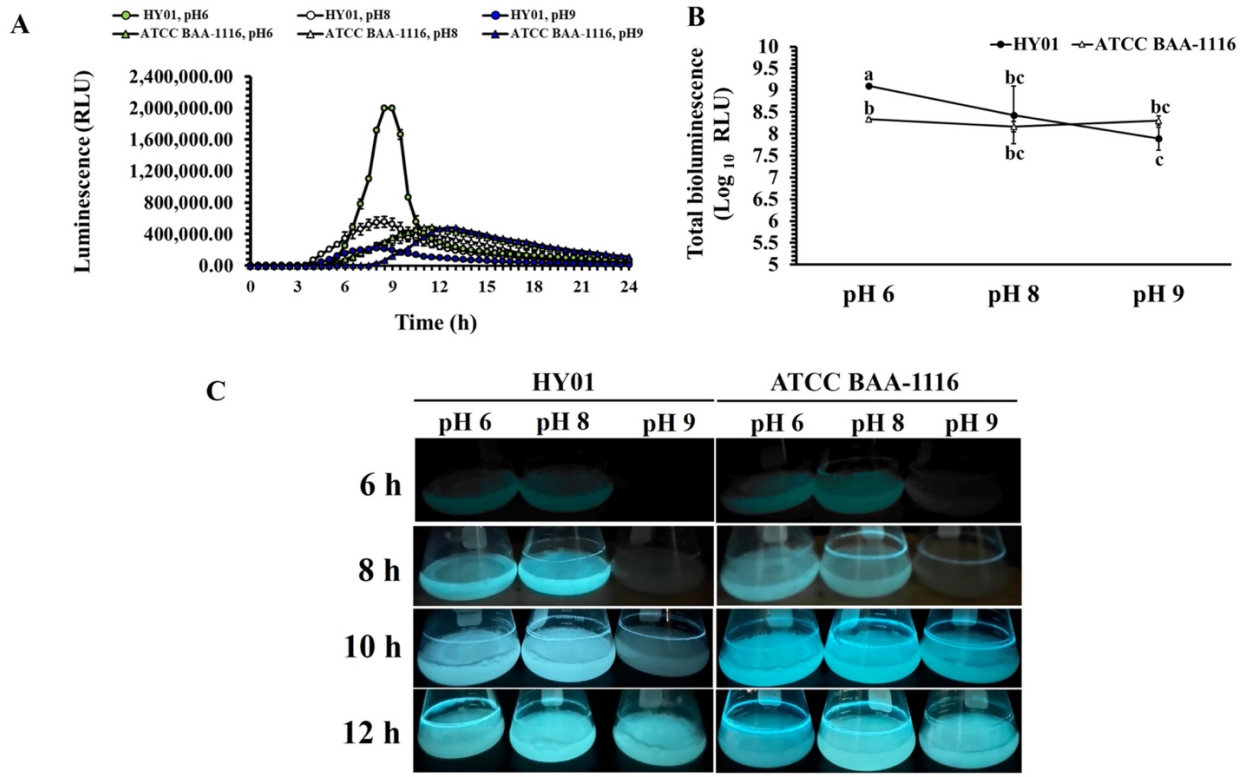

Figure 3 Effect of acidic and alkaline pH conditions on bioluminescence of shrimp pathogenic *V. campbellii* strain HY01 and non-pathogenic *V. campbellii* strain ATCC BAA-1116. Bioluminescence kinetics of both strains under different pH conditions over a 24-h incubation period (A). Total bioluminescent emission calculated as the area under the curves (B). Bluish-green light emitted by *V. campbellii* strains grown in 30 pL/L LBS adjusted to different pH conditions, captured in a dark room at various incubation times (C). Levene's test and Shapiro-Wilk test indicated equal variances and normal distributions (p-value ≥ 0.05). The lowercase letters represent the statistical analysis using Tukey's post hoc test for comparison among groups at p-value < 0.05. Different lowercases denote statistically significant differences, while the same lowercase letters indicate no statistically significant differences.

Biofilm formation, exopolysaccharide production, surface colonization, and motility in *V. campbellii* are disrupted by pH levels.

Environmental stressors, particularly pH, influence the ability of *V. campbelii* to utilize polar flagellum for surface attachment, aggregate into biofilm-like structures, and form multi-layered protective shields, facilitating long-term persistence in adverse habitats. In this study, 48-h cultures of the pathogenic strain HY01 and the non-pathogenic strain ATCC BAA-1116 exhibited the highest biofilm biomass at pH 6. In contrast, alkaline conditions significantly suppressed biofilm formation in both strains (**Figure 4(A)**). CLSM further demonstrated pH-dependent effects on exopolysaccharide production, with strong green fluorescence (indicative of exopolysaccharide binding with concanavalin A) observed at pH 6 for both strains (**Figure 4(B)**). The absence of red fluorescence from PI staining indicated no cell death across conditions. Scanning electron microscopy (SEM) revealed dense biofilm layers on shrimp shells cultured at pH 6, while thinner biofilm layers and scattered cell arrangements were observed under alkaline conditions, suggesting reduced surface colonization (**Figure 4(C)**).

These findings align with previous studies reporting reduced biofilm formation under highly alkaline condition [5,46]. Statistical interaction analysis confirmed that biofilm formation was significantly affected by both pH and bacterial strain (**Table 1**).

Additionally, pH significantly influenced the motility of both *V. campbellii* strains. The largest swimming motility diameter was observed for HY01 cultured in 0.3 % (w/v) LBS agar at pH 6, whereas motility markedly decreased under alkaline conditions.

Similarly, strain ATCC BAA-1116 exhibited reduced motility as pH increased to alkaline level (**Figure 5**). Although 2-way ANOVA indicated no significant interaction between pH and bacterial strains on motility, both pH and strains independently showed significant effects (**Table 1**). Comparative studies in *V. alginolyticus* have shown that flagellar regulatory genes (*flrABC*) exhibit high expression at pH 6 to 8, with decreased expression under extreme acidic or alkaline conditions [47]. Similarly, the Che operon, responsible for adhesion in *V. harveyi*, demonstrated stronger expression at slightly acidic to neutral pH levels compared to pH values above 8 or below 5 [48]. In addition, genes associated with the construction of the polar flagellum in *V. parahaemolyticus* have been shown to be up-regulated under acidic stress conditions [49]. Although our study does not include gene expression data related to biofilm formation and flagellar motility, it can be inferred that alkaline pH conditions may primarily affect genes involved in flagellar assembly and adhesion. This could impair the flagella movement of *V. campbellii* in semi-solid agar. These findings support the idea that pH acts as an external inducer or suppressor of motility-related genes, significantly influencing the physiological responses of *V. campbellii*.

Our findings highlight the impact of external pH conditions on phenotypic expression related to colonization processes in *V. campbellii*. Increased pH levels may hinder flagellar movement and adhesion to surfaces, thereby reducing the bacteirum's ability to produce exopolysaccharides and form biofilms. These disruptions could impair *V. campbellii*'s capacity for infection and colonization in shrimp. Therefore, external pH conditions may serve as an environmental indicator, indirectly estimating the risk of *V. campbellii* distribution and infection in aquaculture ponds.

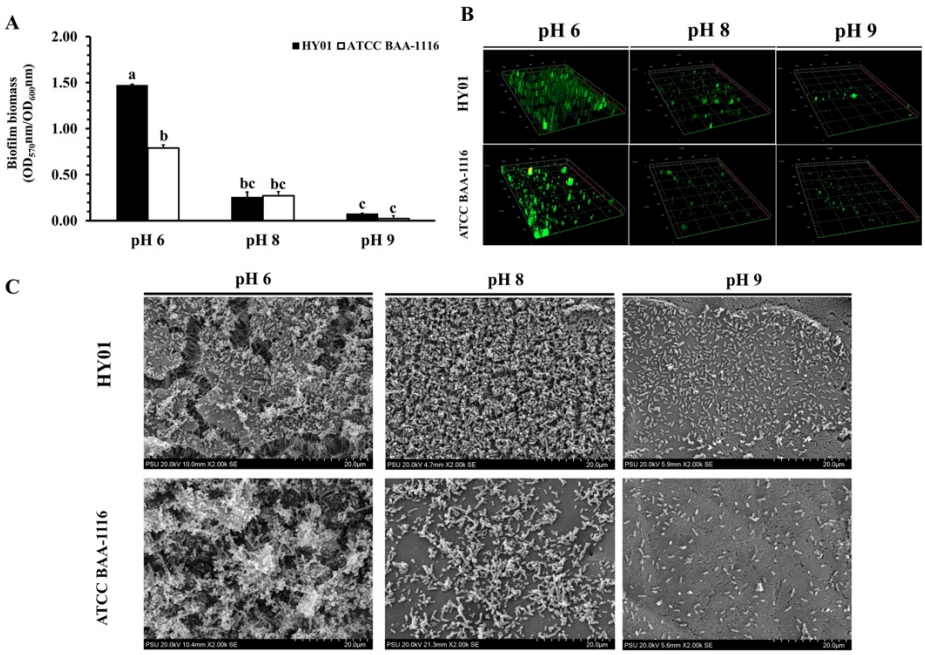

Figure 4 Biofilm formation ability of the shrimp pathogenic *V. campbellii* strain HY01 and the non-pathogenic *V. campbellii* strain ATCC BAA-1116 under acidic and alkaline conditions. Biofilm quantification for both strains grown at different pH levels was assessed using CV staining (A) and visualized using CLSM (B). Additionally, biofilm structures of *V. campbellii* attached to shrimp shell surfaces under varying pH conditions were observed using SEM (C). Levene's test and Shapiro-Wilk test indicated equal variances and normal distributions (p-value ≥ 0.05). The lowercase letters represent the statistical analysis using Tukey's post hoc test for comparison among groups at p-value < 0.05. Different lowercases denote statistically significant differences, while the same lowercase letters indicate no statistically significant differences.

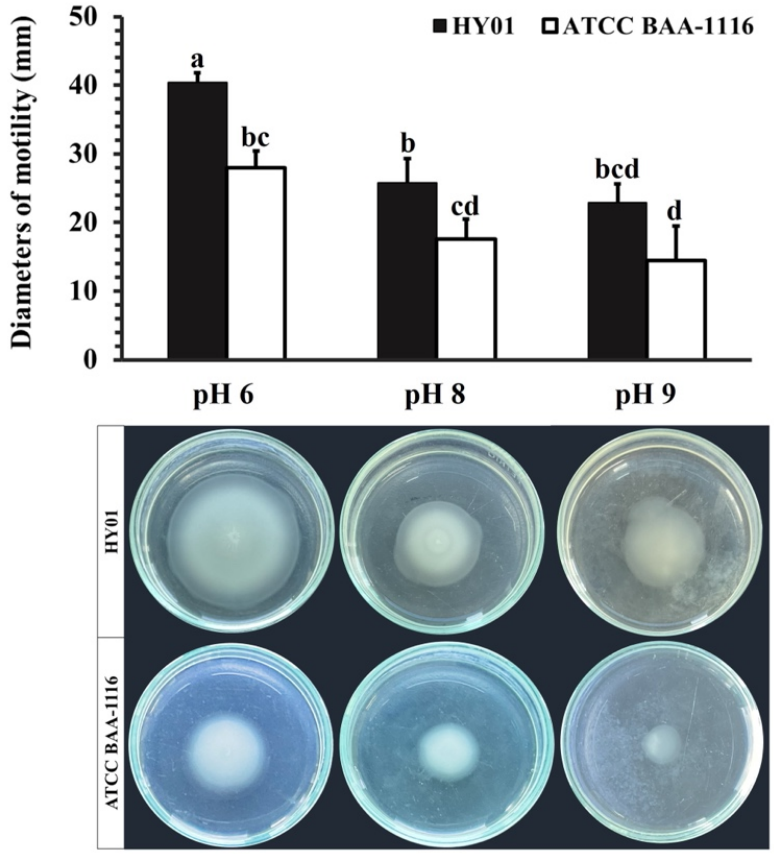

Figure 5 Swimming motility diameters of the shrimp-pathogenic *V. campbellii* strain HY01 and the non-pathogenic *V. campbellii* strain ATCC BAA-1116 grown in semi-solid LBS agar under different pH conditions. Levene's test and Shapiro-Wilk test indicated equal variances and normal distributions (p-value ≥ 0.05). The lowercase letters represent the statistical analysis using Tukey's post hoc test for comparison among groups at p-value < 0.05. Different lowercases denote statistically significant differences, while the same lowercase letters indicate no statistically significant differences.

The production of degradable enzyme in *V. campbellii* is affected by pH levels

The 72-h cultures of the pathogenic strain HY01 and the non-pathogenic strain ATCC BAA-1116 on skim milk agar demonstrated clear proteolytic zones, with variations dependent on pH levels. At pH 6 and 8, the proteolytic zones of both strains showed no significant difference between these conditions. However, at pH 9, proteolytic activity was slightly reduced in both strains, indicating a decrease in their ability to degrade skim milk (**Figure 6**). This finding aligns with prior studies on *V. parahaemolyticus*, where caseinase production significantly declined under basic pH conditions [50]. However, it is important to note that this study involved a preliminary screening of extracellular enzymatic digestion using skim milk agar and demonstrated only the capability of *V. campbellii* to produce caseinase under varying pH conditions. It does not confirm the direct impact of pH on enzyme activity. Previous studies have reported that proteolytic activity is often highest at neutral to slightly alkaline pH levels [51,52]. Statistical analysis indicated no interaction between pH and bacterial strains in affecting enzymatic production; however, each factor (pH and bacterial strain) independently had a significant effect on caseinase production (**Table 1**). Therefore, alkaline conditions hindered proteolytic production, which is crucial for *V. campbellii* to degrade host proteins and facilitate infection. This corresponded with a reduction in biofilm formation, exopolysaccharide production, and swimming motility under alkalinity.

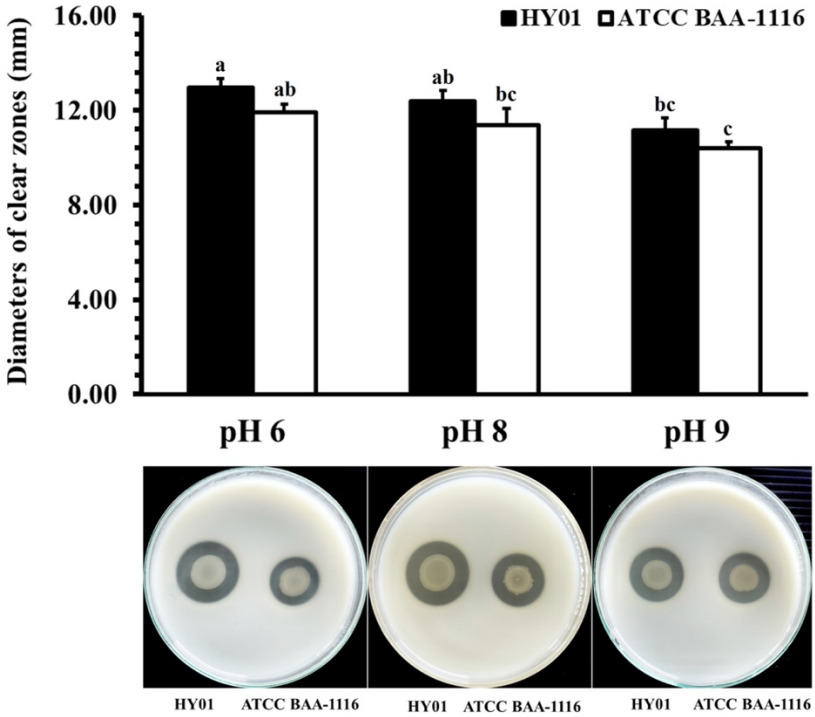

Figure 6 Proteolytic zones of shrimp pathogenic *V. campbellii* strain HY01 and non-pathogenic *V. campbellii* strain ATCC BAA-1116 grown on skim milk agar under acidic and alkaline pH conditions. Levene's test and Shapiro-Wilk test indicated equal variances and normal distributions (p-value ≥ 0.05). The lowercase letters represent the statistical analysis using Tukey's post hoc test for comparison among groups at p-value < 0.05. Different lowercases denote statistically significant differences, while the same lowercase letters indicate no statistically significant differences.

Our study summarized that both pathogenic and non-pathogenic *V. campbellii* strains exhibited greater adaptability under slightly acidic conditions. When the pH of the medium was shifted to more alkaline levels, phenotypic tests clearly showed that pH 6, enhanced bioluminescence, biofilm formation, exopolysaccharide production, and caseinase activity. These enhanced features may facilitate *V. campbellii*'s ability to infect shrimp and possible increase its pathogenicity. This is consistent with previous studies demonstrating that the shrimp pathogenic strain HY01 displayed acidic adaptation at pH 5.0, and acid-adapted HY01 cells exhibited increased pathogenicity in the *Galleria mellonella* [18]. Furthermore, our study revealed the phenotypic mechanisms underlying the pH adaptability of *V. campbellii* strains. By altering the external pH, the bacteria can survive and recover growth, which in turn affects the activation or suppression of various physiological features. This phenomenon needs further investigation through molecular and bioinformatic studies to explore the differences in pH-adaptative mechanisms between pathogenic and non-pathogenic *V. campbellii* strains.

Conclusions

This study highlights the role of external pH as both an environmental stressor and an enhancer of *V. campbellii*'s growth and phenotypic expression. Under pH stress, *V. campbellii* demonstrated the ability to modify its surrounding environment, shifting it toward slightly alkaline levels, which closely resemble its natural habitats. The pathogenic strain HY01 exhibited remarkable adaptability across a broad range of pH conditions, outperforming the non-pathogenic strain in growth, luminescence, biofilm formation, exopolysaccharide production, motility, and caseinase production. The findings underscore the pathogenic strain's resilience to slightly acidic conditions, which may be associated with gradual pH decreases caused by ocean acidification, and its ability to thrive in extreme alkaline conditions often observed in farmed rearing waters affected by waste accumulation, algal blooms, or seasonal

changes. These adaptive traits highlight the potential for *V. campbellii* to persist and flourish despite pH fluctuations. However, this work provides a comprehensive phenotypic profile of *V. campbellii* under varying pH conditions, it is limited in its ability fully illustrate the adaptative mechanism under pH stress. To address the gap, further research will focus on exploring these mechanisms through molecular studies, laying the foundation for future genetic, transcriptomic, and proteomic studies to uncover the molecular mechanisms underlying its pH response throughout different growth stages. Understanding these environmental adaptations will provide valuable insights for managing aquaculture environments and predicting luminous vibriosis outbreaks under changing climate conditions.

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