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Research Article

Inactivation Kinetics of *Vibrio parahaemolyticus* on Sand Shrimp *(Metapenaeus ensis)* by Cinnamaldehyde at 4°C

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Sand shrimp *(Metapenaeus ensis)*, shrimp shell, and shrimp meat were inoculated with a three-strain cocktail of *Vibrio parahaemolyticus* with or without the natural antimicrobial cinnamaldehyde (2.5 mg/ml) and were, then, stored at 4°C for up to 25 days and 18 inactivation curves were obtained. *V. parahaemolyticus* were inactivated down to the minimum level of detection (2.48 log CFU/g) on thiosulfate citrate bile salts sucrose agar (TCBS) plates within 7 and 10 days with low and high densities of *V. parahaemolyticus* inoculation, 4.5 log CFU/g and 8.2 log CFU/g, respectively. With adding cinnamaldehyde, the inactivation process of *V. parahaemolyticus* with low populations, 4.5 log CFU/g, lasted for only 4 days. Therefore, cinnamaldehyde inactivated cells faster as expected. However, unexpectedly, in shrimp meat cases, cells have much more persistence of over even 25 days before entering the minimum level of detection both with and without cinnamaldehyde treatment. Therefore, a hypothesis was formed that when cells kept in cold environments (4°C) after several days recovered to up to 10^3–10^4 CFU/g towards the end of the experiments and with starvation (shell and shrimp studies), cells might render a viable but nonculturable (VBNC) state.

1. Introduction

Vibrio parahaemolyticus is a halophilic bacterium and a human pathogen, naturally occurring and widely distributed in tropical and temperate aquatic environments worldwide [1, 2]. It is the leading cause of seafood-associated bacterial gastroenteritis in the world, causing one of the most severe forms of this disease, and is often associated with the consumption of raw, undercooked, or mishandled seafood [3]. In previous studies [4], *V. parahaemolyticus* experiments have usually been carried out on shellfish, oysters, and mussels. However, few data are available for crustaceans, despite the popularity of crabs and shrimp and their rising consumption worldwide [5, 6]. Shrimp are particularly known as highly perishable products because, unlike other crustaceans (crabs, lobsters), which can be kept alive until processing, shrimp die soon after being caught [7]. With increases in the international trade of shrimp, the consumption of raw or lightly cooked shrimp and the interest in *V. parahaemolyticus* have greatly increased in recent years [8].

The ubiquitous nature of *Vibrio* species in marine environments makes it impossible to obtain shrimp completely free of these bacteria. Several species of shrimp, such as sand shrimp *(Metapenaeus ensis)* are generally sold live at markets in China and could be the origin of cross-contamination to other seafood [9]. Recently, foodborne diseases by *V. parahaemolyticus* have occurred with the emergence of a new pandemic clone creating outbreaks of unprecedented magnitude spread over large areas [10–15]. Sujeewa et al. [16] studied the prevalence of virulent strains of *V. parahaemolyticus* in frozen ready-to-eat (RTE) shrimp for human consumption and found that 7 to 8% of samples tested positive for the tdh and trh genes in countries such as Malaysia. Therefore, these shrimp might have had the

potential to cause *V. parahaemolyticus*-associated illnesses if consumed without further processing, indicating a probable risk to human health [17].

Moreover, due to the vast usage of antibiotics worldwide, the accumulation of antibiotic-resistant bacteria and genes, leading to the treatment failure or longer convalescence in both humans and animals, studies on antibiotic replacement are currently emerging [18]. Use of natural antimicrobials, such as extracts from spices and herbs for the purpose of food preservation or the substitution of antibiotics, has arisen. It has been well documented that essential oils or their active components from wild plants' natural defense systems can act as effective antimicrobials [19]. Cinnamaldehyde is a major active component from cinnamon essential oils and has shown promising antimicrobial capability against a number of food borne pathogens [20, 21]. To date, cinnamaldehyde has been registered by the European Commission and is considered to present no risk to human health [22]; however, there has been limited number of reports on their antimicrobial activity against *V. parahaemolyticus*. Especially in previous studies, Zhang et al. [23] reported that two identical peaks analyzed in a UPLC (Ultra Performance Liquid Chromatography) system appeared with bacteria alone (i.e., *L. monocytogenes* ATCC 19115 and *E. coli*) but disappeared with cinnamaldehyde treatments. The two peaks are suspected to be two identical metabolic products produced by both of the isolates without cinnamaldehyde treatments and these two metabolites may be related to quorum-sensing pathways.

Thus, the purpose of the present study is to evaluate the growth of *V. parahaemolyticus* in sand shrimp *(Metapenaeus ensis)* over a period of approximately ten days of storage at 4°C.

2. Materials and Methods

2.1. Inocula Preparation. Three *V. parahaemolyticus* strains (ATCC 17802, ATCC 33846, and ATCC 33847) were obtained from ATCC (USA) and maintained in nutrient broth (NB, Oxoid) containing 30% glycerol (Sigma) and 3% NaCl [24, Sigma] at −80°C. Experimental inocula for each strain were cultured from five bacterial colonies on nutrient agar (NA, Oxoid) with 3% NaCl and grown to stationary phase in 80 ml NB with 3% NaCl by incubation at 37°C for 24 (±0.5) h to achieve a population density of approximately 9 log CFU/ml.

2.2. Shrimp Sampling Preparation and Storage. Sand shrimp *(Metapenaeus ensis)* were purchased from local wet markets in Shanghai, China. To minimize stress during transport, collected samples were placed inside a 5 L shopping polythene bag filled with seawater from the seafood stand in the market and immediately transported to the laboratory under continuously oxygenated conditions. Twenty-five grams of shrimp, shrimp shell, and shrimp meat (hand peeled aseptically) were then immediately weighed and separately stored in sealed sterile polythene bags (Twirl'em sample bags, Labplas Inc.) at 4°C.

Three ml of each stationary phase *V. parahaemolyticus* strain population was thoroughly mixed. The mixed cells were serially diluted in peptone physiological saline (pps) (0.85% NaCl, 0.1% peptone (Oxoid)) and 0.1 ml of the prepared culture was inoculated onto presorted 25 g samples of shrimp, shrimp skins, and shrimp meat, respectively, and mixed thoroughly, to obtain an initial concentration of about 4.5 or 8.2 log CFU/g, respectively. After 4.5 log CFU/g inoculation, where it was necessary, samples were treated with a twofold serial diluted natural antimicrobial, cinnamaldehyde (Sigma), to achieve the final concentration of 2.5 mg/ml. Noninoculated packs of shrimp, shrimp skins, and shrimp meat in which *V. parahaemolyticus* counts were below the detection limit (2.48 log CFU/g) (data not shown) were used as a control. At appropriate time intervals, shrimp samples were aseptically taken out of the packs for microbiological analyses. Three packs of shrimp were used to obtain triplicate analysis per sampling time point.

2.3. Microbiological Analysis. The inactivation of *V. parahaemolyticus* and total viable counts was estimated by culture-based enumeration immediately before and after inoculation and at selected intervals up to 26 days of storage. Specifically, 225 ml of pps with 3% NaCl and a 25 g sample were transferred aseptically into a stomacher bag. The mixture was homogenized for 2 min by lightly pummeling with a BagMixer® (Interscience France). One hundred microliters was removed and tenfold serial dilutions of pps were prepared as needed. One hundred microliters of the appropriate dilutions was surface-plated using a sterile cell spreader onto NA supplemented with 3% NaCl for enumeration of total bacteria and thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid) to enumerate *Vibrio* species, respectively. Plates were incubated at 37°C for 24 (±0.5) h. Colony forming units were quantified and identified by the VITEK® 2 microbiological identification system (BioMérieux). Inactivation curves were constructed by plotting $\log_{10}$ CFU/ml against time [25].

2.4. Statistical Analysis. Assuming log-linear inactivation kinetics, the rate of inactivation of *V. parahaemolyticus* was calculated from each of the 27 survival curves by linear regression analysis using Microsoft Excel. The mean *D*-value for each survival curve was determined by each inactivation rate. The significance differences between the *D*-values for each curve were evaluated by a Student's two-tailed *t*-test assuming unequal variance using Microsoft Excel. Differences were considered to be significant when $p > 0.05$.

3. Results and Discussion

3.1. V. parahaemolyticus Inactivation in Shrimp with Low Density of Inoculation and Cinnamaldehyde. Figures 1(a)–1(c) show the changes of *V. parahaemolyticus* population density after inoculation with 4.5 log CFU/ml. The total cell density for all meat, shell, and shrimp samples for Figures 1(a)–1(c) gradually increased from 10^5–10^6 to 10^6–10^7 and remained constant throughout the balance of the study. *V. parahaemolyticus* decreased dramatically from 10^4–10^5 CFU/ml to the minimum level of detection (2.48 log CFU/ml) in 7 days for all samples; however, an interesting phenomenon

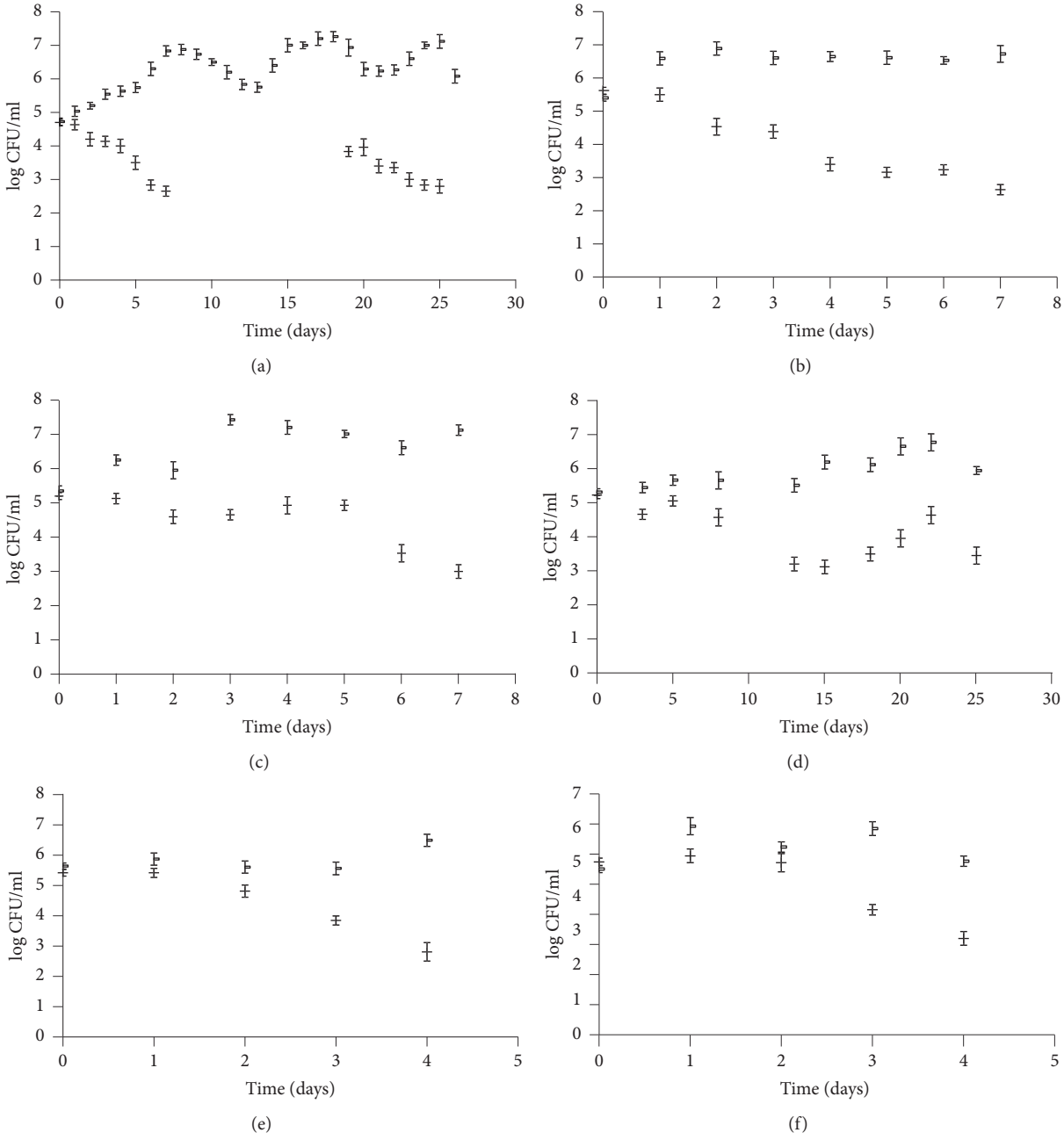

Figure 1: The growth of *V. parahaemolyticus* was estimated by culture-based enumeration immediately before and after inoculation with an initial population of 4.5 log CFU/ml, or 2.5 mg/ml cinnamaldehyde treatment, and at intervals throughout 26 days of storage. Growth curves were constructed by plotting log CFU/ml against time; Figure 1(a) for shrimp meat, Figure 1(b) for shrimp shell, Figure 1(c) for whole shrimp, Figure 1(d) for shrimp meat with cinnamaldehyde treatment, Figure 1(e) for shrimp shell with cinnamaldehyde treatment, and Figure 1(f) for whole shrimp with cinnamaldehyde treatment. Colony forming units were quantified with total bacterial count on NA (−) and *V. parahaemolyticus* (+) on TCBS. Bars represent the standard deviation of three independent samples.

occurred at day 19 only for meat samples, when *V. parahaemolyticus* recovered to 10^4 CFU/ml, but dramatically dropped again to minimum level of detection by day 25.

For Figures 1(d)–1(f), cinnamaldehyde treatment was applied after inoculation with 4.5 log CFU/ml of cells. Total cell populations remained constant at 10^6–10^7 CFU/ml, which is similar with control. For control, the total cell densities for all meat, shell, and shrimp samples were always within 10^6–10^7 CFU/ml, while there was no *V. parahaemolyticus* detected in natural *Vibrio* species in shrimp (data not shown). For cinnamaldehyde treatment, *V. parahaemolyticus* decreased from 10^5 CFU/ml to 10^3 CFU/ml for meat samples

at day 15 and then gradually increased to 10^4 CFU/ml at day 22 and dropped to minimum level of detection at day 25. For both shell and shrimp samples, *V. parahaemolyticus* gradually decreased from 10^5 CFU/ml to minimum level of detection at day 4. Obviously, for meat samples *V. parahaemolyticus* was persistent for the entire study of 25 days. These results were expected as cinnamaldehyde treatment was initially applied to inhibit *V. parahaemolyticus* from growth, and these results demonstrate that the cells can survive longer under an assumed lethal temperature of 4°C.

Therefore, as observed from Figures 1(a)–1(f), 4°C fully stopped *V. parahaemolyticus* from growth with or without cinnamaldehyde treatment. Chiu et al. [26] discovered similar phenomenon that when *V. parahaemolyticus* was cultured at a low temperature, slow growth was observed at 15°C but growth ceased at 10° or 4°C and the viability of cells rapidly declined at 4°C. Nevertheless, when cold shock was applied to the pathogen in a carbon starvation medium, the cells survived at 4°C, which is opposite from the current study that, in shrimp meat samples with cinnamaldehyde treatment, *V. parahaemolyticus* persists longer.

A possible explanation for the longer survival colonies might have to do with TCBS agar. It is documented that there are several limitations associated with the agar for the correct identification of *V. parahaemolyticus* [11, 27]. On TCBS, *V. parahaemolyticus* shows morphology and characteristics similar to other *Vibrio* spp. such as *V. vulnificus, V. mimicus,* and *V. fluvialis*, while *V. parahaemolyticus* and *V. vulnificus* form 3–5 mm and 2-3 mm green colonies on TCBS, respectively [28]. In addition to this lack of specificity, the limited selectivity of the medium allows for the overgrowth of other bacteria that predominate in the environment, thereby masking the presence of *V. parahaemolyticus* and complicating the identification of *Vibrio* colonies [29]. In the current study, through biochemical confirmation via the VITEK 2 microbiological identification systems green colonies on TCBS can be identified; however, it is impossible to test each and every green colony in this study. Therefore, selected number of green colonies from each TCBS plate were tested and then identified to be *V. parahaemolyticus*.

A possible explanation for cinnamaldehyde treatment against *V. parahaemolyticus* was reported by Moleyar and Narasimham [30] in that 4% w/v salt in agar did not improve the antibacterial activity of cinnamaldehyde against Gram-positive and Gram-negative bacteria. Also, a mixture of cinnamaldehyde and eugenol at 250 and 500 Ag/ml, respectively, completely inhibited the growth of *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp., and *Enterobacter* sp. for more than 30 days, whereas when the compounds were applied individually, growth was not inhibited [31]. Zhang et al. [23] reported that two identical peaks appeared with bacteria alone (i.e., *L. monocytogenes* ATCC 19115 and *E. coli*) but disappeared with cinnamaldehyde treatments by the UPLC (Ultra Performance Liquid Chromatography) system, which is different from the other three natural antimicrobials, namely, thymol, eugenol, and carvacrol. The two peaks are suspected to be two identical metabolic products related to quorum-sensing pathways [32], since Pei et al. [18] hypothesized that the arbonyl group on cinnamaldehyde may adhere to proteins to prevent the action of amino acid decarboxylases [33]; Niu et al. [34] reported that cinnamaldehyde would influence signaling pathways of quorum sensing by interfering with the binding of 3-hydroxy-C4, 3-oxo-C6-HSL, and AI-2 synthetic pathway and Brackman et al. [35] discovered that cinnamaldehyde interferes with AI-2 based quorum sensing by decreasing the DNA-binding ability of LuxR to bind to its target promoter sequence.

However, as observed in this study, cinnamaldehyde appeared to support *Vibrio* survival and culturability in shrimp meat samples. A possible explanation might be due to antibiotic abuse in sand shrimp farming, as the natural antimicrobial cinnamaldehyde may lose its ability to inhibit *V. parahaemolyticus* from growth since *V. parahaemolyticus* may have already developed antibiotic resistance ability to survive antibiotic and natural antimicrobial interactions during inactivation process. Another possible explanation might be that cells incubated at 4°C might enter the viable but nonculturable (VBNC) state, while cells in the first week of storage may have been a mixture of culturable and VBNC cells [36, 37]. The mechanism of cinnamaldehyde that triggered the cellular recovery from VBNC state is unknown and still needs further investigation. Wesche et al. [38] explained that the alternate sigma factor, when triggered in bacterial pathogens under stress, may induce cellular survival or cross protection, enabling cells to resist other environmental stresses. It is possible that cinnamaldehyde could have triggered this or similar stress-response mechanisms, leading to cellular "hardening," which could have promoted survival at the 4°C storage temperature.

3.2. V. parahaemolyticus Inactivation in Shrimp with High Density of Inoculation. For Figures 2(a)–2(c), total counts remained constant at 10^8–10^9 CFU/ml. *V. parahaemolyticus* gradually decreased from 10^8 CFU/ml to minimum level of detection at day 10 for all meat, shell, and whole shrimp samples. Compared with Figures 1(a)–1(c), *V. parahaemolyticus* had longer survival duration under storage at 4°C, except for meat samples. The 8.2 log CFU/ml inoculation level, when compared with the 4.5 log CFU/ml inoculation level and 2.5 mg/ml cinnamaldehyde treatment at 4°C, allowed greater *V. parahaemolyticus* persistence for the lower inoculum level, especially for meat samples. This observation reinforces the previous findings that cells under these environment stresses may have entered VBNC state.

In the present study, experiments were, in parallel, divided into three categories, shrimp meat, shrimp shell, and whole shrimp (Table 1). The *D*-value was calculated for each *V. parahaemolyticus* inactivation curve. For low density inoculation (4.5 log CFU/ml), *D*-values were 24.69 ± 0.65, 2.22 ± 0.02, and 3.79 ± 0.17 (mean ± standard deviation) for shrimp meat, shrimp shell, and whole shrimp, respectively. While for both low density inoculation and cinnamaldehyde treatment (2.5 mg/ml), *D*-values were 14.16 ± 0.55 for shrimp meat, 1.48 ± 0.04 for shrimp shell, and 1.66 ± 0.14 for whole shrimp. However, when samples were inoculated with high density *V. parahaemolyticus* (8.2 log CFU/ml), *D*-values substantially decreased to 2.49 ± 0.05 for shrimp meat, 2.58 ± 0.07 for shrimp shell, and 1.79 ± 0.04 for whole shrimp.

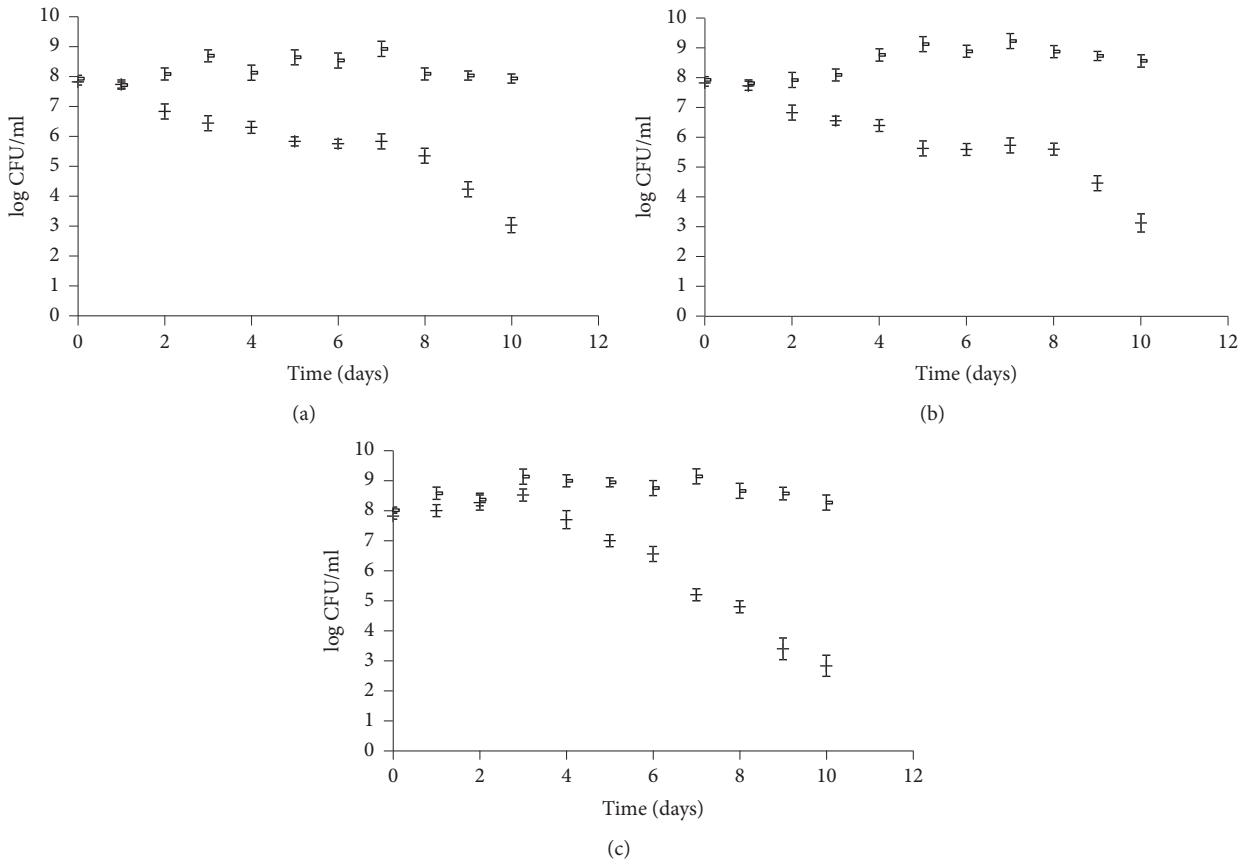

Figure 2: The growth of *V. parahaemolyticus* was estimated by culture-based enumeration immediately before and after inoculation at 8.2 log CFU/ml and at intervals throughout 10 days. Growth curves were constructed by plotting log CFU/ml against time; Figure 2(a) for shrimp meat, Figure 2(b) for shrimp shell, and Figure 2(c) for whole shrimp. Colony forming units were quantified with total bacteria count on NA (−) and *V. parahaemolyticus* (+) on TCBS. Bars represent the standard deviation of three independent samples.

Differences in the *D*-value of each inactivation curve at a given condition were not significant ($p < 0.05$).

Nutritionally, by weight, the order of samples would be meat > whole shrimp > shell. Thus, cells entering an environment at 4°C with different nutrient levels might induce starvation within shrimp shell and whole shrimp, therefore entering the VBNC state. Kaneko and Colwell [39] reported that *V. parahaemolyticus* inhabits warm seawater and marine animals; thus, when the temperature of the seawater is less than 13–15°C, *V. parahaemolyticus* is rarely isolated and the cells under these conditions are suggested to be VBNC. Furthermore, it has been concluded that the VBNC state in this pathogen is induced by incubation at a low temperature in a nutrient-limited medium [40–42] and resuscitated by a temperature upshift treatment [36].

Entering the VBNC state of *V. parahaemolyticus* described herein is a different response from the single stress response to starvation or cold shock. *V. parahaemolyticus* in both the VBNC state [42, 43] and the starved state [44, 45] exhibited enhanced stress resistance and similar changes in cell shape, but the main difference between these two states was in culturability. Thus, induction of the VBNC state by low-temperature incubation in a nutrient-limited medium is a unique response. To support the hypothesis that the VBNC state is regulated by a genetic mechanism, an effective approach would be to isolate mutants that fail to enter the VBNC state; nevertheless, no such mutants have been identified [46]. The preservation procedure known to inhibit *V. parahaemolyticus* from entering VBNC state, the so-called starvation-induced maintenance of culturability

Table 1: The means of *D*-values for 27 *V. parahaemolyticus* inactivation curves of both low and high densities inoculated at 8.2 and 4.5 log CFU/ml, respectively, and low density inoculation with cinnamaldehyde treatment, 2.5 mg/ml.

D-value	Shrimp meat	Shrimp shell	Whole shrimp
Low density inoculation	24.69 ± 0.65	2.22 ± 0.02	3.79 ± 0.17
Cinnamaldehyde treatment	14.16 ± 0.55	1.48 ± 0.04	1.66 ± 0.18
High density inoculation	2.49 ± 0.05	2.58 ± 0.07	1.79 ± 0.04

D-value = mean ± SD (standard deviation); *D*-value was calculated by linear regression to each inactivation curve; *D*-value unit of measurement is days.

(SIMC) effect [47], which has been demonstrated in several bacteria [48–50] has been investigated. Thirteen proteins, including the peroxiredoxin AhpC, were enhanced in the induction period or downregulated in prestarved cultures which inhibited the entry of exponential phase cells into the VBNC state. Expression of mreB in *V. parahaemolyticus* under various environmental stresses, was investigated [26]. Under cold shock or the induction of the VBNC state, the mreB level remained relatively high initially and declined thereafter. The mreB level was elevated in cells that were moved back to the nutrient rich medium from starvation as well as in the temperature upshifted VBNC cells. Toxin genes (thermolabile hemolysin and thermostable direct hemolysin) are also known to be expressed in the VBNC state of *V. parahaemolyticus* [51]. However, the metabolic responses and/or genetic control of VBNC state have not been clarified.

4. Conclusions

The inactivation of *V. parahaemolyticus* in shrimp was investigated in this study. Fifty-four inactivation curves were generated. Typical green colonies were picked and underwent VITEK 2 microbiological identification system testing and were confirmed as *V. parahaemolyticus*. *V. parahaemolyticus* was inactivated within 10 days to the minimum level of detection on TCBS agar, except for meat samples. There was no *V. parahaemolyticus* detected in natural *Vibrio* species in sand shrimp, cinnamaldehyde inactivated cells faster as expected, and *D*-values were substantially decreased for high density inoculation of *V. parahaemolyticus,* compared with low density inoculation or with cinnamaldehyde treatment, especially for shrimp meat samples. Therefore, cells undergoing starvation in shell and whole shrimp held at 4°C may have been induced into the VBNC state. Although the literature documents a genetic control mechanism, including change in cellular shape and protein expression profile, the exact mechanism of cells entering VBNC state remains unclear.

Conflicts of Interest

The authors declare that no competing financial interests exist.

Acknowledgments

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