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Fate of *Vibrio parahaemolyticus* on shrimp after acidic electrolyzed water treatment

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ABSTRACT

The objective of this study was to investigate the fate of *Vibrio parahaemolyticus* on shrimp after acidic electrolyzed water (AEW) treatment during storage. Shrimp, inoculated with a cocktail of four strains of *V. parahaemolyticus*, were stored at different temperatures (4–30 °C) after AEW treatment. Experimental data were fitted to modified Gompertz and Log-linear models. The fate of *V. parahaemolyticus* was determined based on the growth and survival kinetics parameters (lag time, λ; the maximum growth rate, μ_{max}; the maximum growth concentration, D; the inactivation value, *K*) depending on the respective storage conditions. Moreover, real-time PCR was employed to study the population dynamics of this pathogen during the refrigeration temperature storage (10, 7, 4 °C). The results showed that AEW treatment could markedly ($p < 0.05$) decrease the growth rate (μ_{max}) and extend the lag time (λ) during the post-treatment storage at 30, 25, 20 and 15 °C, while it did not present a capability to lower the maximum growth concentration (D). AEW treatment increased the sensitivity of *V. parahaemolyticus* to refrigeration temperatures, indicated by a higher ($p < 0.05$) inactivation value (*K*) of *V. parahaemolyticus*, especially for 10 °C storage. The results also revealed that AEW treatment could completely suppress the proliferation of *V. parahaemolyticus* in combination with refrigeration temperature. Based on above analysis, the present study demonstrates the potential of AEW in growth inhibition or death acceleration of *V. parahaemolyticus* on seafood, hence to greatly reduce the risk of illness caused by this pathogen during post-treatment storage.

1. Introduction

Vibrio parahaemolyticus is recognized as the most common food-borne pathogen, and also considered to be the leading cause of seafood-derived illness in China (Liu et al., 2012; Mao et al., 2010; Xu et al., 2013). This pathogen exists in a variety of raw seafood, and consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, etc. (Su and Liu, 2007; Xie et al., 2012).

Shrimp is one of the most important fishery products in South and Southeastern parts of Asia, as well as an important economy characteristic in these areas (Lin et al., 2013; Xu et al., 2013). However, the contamination rate of *V. parahaemolyticus* has increased in shrimp culture environment year after year, and the natural germ-carry rate of shrimp could reach 90% in the warm seasons. Thus, *V. parahaemolyticus* has become the predominant harmful factor of raw shrimp (Pu et al., 2013; Su and Liu, 2007). Additionally, cooked shrimp are often picked by hand, and also can be easily contaminated with *V. parahaemolyticus* through bad manufacturing practices and poor personal hygiene (Liu et al., 2006; McCarthy, 1997; Wang et al., 2014) during each course including storage, transportation and distribution (Dupard et al., 2006; Gudbjorndottir et al., 2005). Moreover, risk assessment of *V. parahaemolyticus* on cooked black tiger shrimp has been conducted in Malaysia in 2008 and 2012, and the results showed that consuming cooked shrimp could cause illness related with *V. parahaemolyticus* (Sani et al., 2012, 2008). Therefore, food scientists and food industry are searching for novel non-thermal methods that could destroy undesired microorganisms with less adverse effects on products (Ju et al., 2008; Wang et al., 2014).

Several studies have been performed on non-thermal methods for decontaminating bacteria on fresh produce, such as organic acids, compounds of chlorine, pulsed electric field (PEF), etc. (Ding et al., 2010; Huang et al., 2014; Pipek et al., 2006). Acidic electrolyzed water (AEW) is regarded as one of the most promising, with a high efficacy for inactivating food-borne pathogens (Ding et al., 2010; Wang et al., 2014). It has been demonstrated that AEW has a strong disinfectant effect on *V. parahaemolyticus*. Ren and Su (2006) investigated the effects of electrolyzed oxidizing water treatment on reducing *V. parahaemolyticus* in raw oysters. The results showed that holding oysters inoculated with *V. parahaemolyticus* in AEW for 4 to 6 h resulted in

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significant reductions of *V. parahaemolyticus* by 1.13 Log_{10} MPN (Most Probable Number)/g. Chiu et al. (2006) reported that populations of *V. parahaemolyticus* on stainless steel, ceramic tile, and plastic cutting boards were reduced by AEW treatment. Quan et al. (2010) showed that reductions of *V. parahaemolyticus* were obtained with weakly acidic electrolyzed water (WAEW) treatment. Furthermore, the results from the study of our group demonstrated that combining basic electrolyzed water pretreatment and mild heat could greatly enhance the efficacy of AEW against *V. parahaemolyticus* on shrimp (Xie et al., 2012).

Although AEW has been demonstrated to be effective in inactivating pathogenic microorganisms in foods, it is usually not able to provide complete inactivation on food-borne pathogens, with the surviving population being, most likely, sub-lethally injured (Fang et al., 2013; Moosekian et al., 2014; Silva-Angulo et al., 2014). Thus, some important aspects should be considered as emerging risks in the use of non-thermal technologies including AEW. These aspects are the changes that could take place after sub-lethal injury of pathogens, such as changes in the resistance to stressing treatment. Once foods are removed to a favorable storage conditions, injured cells could undergo cellular repair and proliferate when their recovery was completed. Studies have shown that sub-lethally injured pathogens could proliferate to microbiologically hazardous levels during the sprouting process, when water and nutrients are plentiful (Ariefdjohan et al., 2004; Silva-Angulo et al., 2014). Therefore, it is imperative to investigate the fate of pathogens in foods after non-thermal technologies treatment, including physiological adjustment period of cells prior to growth, exponential growth rate, maximum population density in stationary growth phase and inactivation rate (Silva-Angulo et al., 2014), with the purpose of taking measures to reduce the risk of illness caused by pathogens.

Although some studies have been performed to study the efficiency of AEW on inactivating *V. parahaemolyticus* on seafood, no studies were reported on investigating the fate of *V. parahaemolyticus* on seafood during storage after AEW treatment. Thus, shrimp, a typical representative of seafood, were chosen as the experimental subject in this study. The objective was to investigate the fate of *V. parahaemolyticus* on shrimp during storage after AEW treatment by predictive microorganisms models, and hence to provide more systematic information for ensuring other seafood safety after AEW treatment in food industry in the future.

2. Materials and methods

2.1. Bacterial strains and culture preparation

A four-strain cocktail of *V. parahaemolyticus* strains (ATCC 17802; ATCC 33847; F, shrimp isolate; F18, river prawn isolate) was used in the study. Each strain for *V. parahaemolyticus* (stored in 25% glycerol at −20 °C) was separately enriched in tryptic soy broth (TSB, Beijing Land Bridge Technology Company Ltd., Beijing, China) plus 3% NaCl and incubated at 37 °C for 18–20 h. One hundred μl of each strain was transferred to a second tube with 10 ml TSB and incubated for 10–12 h at 37 °C, resulting in early-stationary-phase culture. Enriched cultures were pooled into a sterile centrifuge tube and centrifuged at 3000 *g*, 15 °C for 10 min (Centrifuge 5417R, eppendorf, Germany). The resulting cell pellet was washed with phosphate-buffered saline (PBS) once. Pelleted cells were re-suspended in phosphate-buffered saline (PBS) to produce a multistrain cocktail of ~9 Log_{10} CFU/ml.

2.2. Preparation of shrimp samples

Live shrimp (10 ± 1 g per sample) were purchased from a local supermarket in Shanghai, PR China and stored at −20 °C before treatment.

Shrimp were thawed firstly and then exposed to boiling water (plus with 2.5% NaCl) bath for 20 min to inactivate the native bacteria of shrimp according to the methods of Xie et al. (2012). Then shrimp were transferred into a biosafety hood quickly until the temperature cooled to room temperature (22 ± 2 °C) before subsequent treatment.

2.3. Preparation of acidic electrolyzed water (AEW)

Acidic electrolyzed water (AEW) was prepared according to study done by Wang et al. (2014). Briefly, AEW was generated by electrolyzing 0.15% sodium chloride solution using AEW generator (FW-200, AMANO, Japan) with an electrochemical cell where the anode and cathode are separated by a diaphragm. The generator was allowed to run for 15 min with the amperage setting as 10 A before collecting water. The solutions were placed into a water bath to reach the treatment temperature. The pH and oxidation reduction potential (ORP) values were measured using a dual scale pH/ORP meter (Mettler-Toledo, Switzerland). The available chlorine concentration (ACC) in AEW was determined by a colorimetric method using a digital chlorine test kit (RC-2Z, Kasahara Chemical Instruments Corp., Saitama, Japan). All measurements were carried out in triplicate. The AEW had a range of pH 2.36, ORP 1173.7, and ACC 66 ppm.

2.4. Experimental procedure

The treatment time and temperature of AEW were determined by previous study (Wang et al., 2014) and they were 2.5 min and 55 °C, respectively.

The experimental protocol was based on previous study by Ding et al. (2010). Briefly, the multistrain cocktail with adjusted concentration was added into 500 ml sterile 2.5% saline solution to obtain the inoculum solution. Shrimp was immersed in the inoculum solution and shaken for 30 min. The shrimp were then removed from inoculum suspension and air-dried in a biosafety hood for 20 min. The final concentration range of *V. parahaemolyticus* inoculated on shrimp was 6.5–7.6 Log_{10} CFU/g. Subsequently, each five samples of shrimp was immersed in 500 ml of AEW at the ratio 1:10 (w/v) for 2.5 min. After treatment, all samples were placed in a sterile stomacher bag filling with 500 ml neutralizing agent (PBS containing 0.8% $Na_2S_2O_3$) to stop the bactericidal effects of AEW. Inoculated samples treated with sterile 0.85% saline solution were used as control.

After AEW treatment, shrimp samples with an even distribution of 3.6 Log_{10} CFU/g of *V. parahaemolyticus* were stored at 30 °C, 25 °C, 20 °C and 15 °C, while shrimp samples with 4.7 Log_{10} CFU/g on average were stored at 10 °C, 7 °C and 4 °C in low temperature incubators with high precision (model MIR 154; Sanyo Electric Co.). At appropriate time intervals, shrimp samples were sampled randomly and mixed with 90 ml of sterile 0.85% physiological saline solution, then homogenized for 2 min in a stomacher (BagMixer400VW, Interscience, France) prior to plating onto TCBS medium (thiosulfate–citrate–bile salts–sucrose, TCBS, Beijing Land Bridge Technology Company Ltd., Beijing, China). Generally, lower temperatures resulted in longer sampling intervals, while shorter intervals were chosen for higher temperatures. Colonies were counted after the plates were incubated at 37 °C for 18 h. Uninoculated cooked shrimp yielded no colonies for either of microorganisms on the agar (TCBS). Two trials with two replicates per trial in each storage condition were done.

2.5. SYBR green real-time PCR assay

Real-time PCR assay was performed according to the protocol of Ye et al. (2012) with a slight modification. Two ml aliquots were transferred into 2 ml sterile tubes. The tubes were centrifuged (Eppendorf, Germany) at 200 *g*, 4 °C for 1 min. The supernatant (1 ml) was aseptically transferred into a 1.5 ml sterile centrifuge tube and a further centrifugation was carried out at 12,000 *g*, 4 °C for 2 min. The pellet was stored at −80 °C before extracting nucleic acids.

Bacterial DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd., China) according to the manufacturer's instruction. To construct standard curves, DNA was extracted from strains suspensions (10^9 CFU/ml), then DNA was 10-fold serially diluted by Easy Dilution (TaKaRa Biotechnology Dalian Co.,

Ltd., China) with concentration ranging from 10^9 to 10^3 CFU/ml. A linear relationship was produced by plotting the Log_{10} CFU/ml against the C_T values. Real-time PCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche Co., Switzerland) using the primers *tlh*-f (ACT CAA CAC AAG AAG AGA TCG ACA A) and *tlh*-r (GAT GAG CGG TTG ATG TCC AA). PCR amplification was performed in a final volume of 20 μl including 2 μl of template DNA, 10 μl of SYBR Green, 1.5 μl (10 mM concentration) of each primer, and 5 μl RNase Free ddH_2O (Shanghai Life feng Biotechnology Co., Ltd., China). PCR program was conducted in ABI Prism 7500 (Applied Biosystems, USA) sequence detection system with the following parameters: 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Fluorescent signals were collected at the extension step. A no-template negative control was included in each run.

2.6. Determination of fate of pathogens on shrimp during post-AEW storage

Describing the fate of AEW-treated *V. parahaemolyticus* on shrimp was divided into two parts in this study. In the first part, modified Gompertz model (Eq. (1)) (Alonso-Hernando et al., 2013; Gibson et al., 1987) was employed to fit the growth curves representing the viable counts (Log_{10} CFU/g) of *V. parahaemolyticus* on shrimp stored at 30 °C, 25 °C, 20 °C and 15 °C, due to its smallest values of AIC (Akaike's information criterion) (unpublished results) comparing with Logistic model (Gibson et al., 1988) and Baranyi model (Baranyi and Roberts, 1994) widely used.

$$N_t = N_0 + A \times \exp\left\{-\exp\left[\frac{\mu \times e}{A}(\lambda - t)\right] + 1\right\} \quad (1)$$

where N_t is the Log_{10} CFU/g of cell concentration at time, t; N_0 is the initial concentration (Log_{10} CFU/g) fitted by the model; A = the increase in bacterial concentration from inoculation to the stationary phase (D), and N_0 = the upper asymptotic curve (concentration of bacteria in the stationary stage, D)-A. λ is the lag phase (h); μ is the maximum growth rate (Log_{10} CFU/g/h).

In the second part, Log-linear model (Eq. (2)) was chosen to fit the data to describe the survival of *V. parahaemolyticus* on shrimp stored at 10 °C, 7 °C and 4 °C after AEW treatment, due to the simplicity and good adequacy of the model based on the parameters in Table 1.

$$Log(S_t) = Log(S_0) + K \times t / \ln(10) \quad (2)$$

where t is time in hours; $Log(S_t)$ is the concentration (Log_{10} CFU/g) of *V. parahaemolyticus* on shrimp at time t; $Log(S_0)$ is the population at time 0 h (Log_{10} CFU/g); and K is related to inactivation rate (Log_{10} CFU/g/h) (Pérez-Rodríguez et al., 2013; Santillana Farakos et al., 2013).

Table 1
The statistical indexes for the modified Gompertz model and log-linear model.

Temperature (°C)	AEW treatment[a]				Control[b]			
	R^2	RMSE	A_f	B_f	R^2	RMSE	A_f	B_f
30	0.983	0.344	1.06	1.00	0.997	0.100	1.06	1.00
25	0.997	0.170	1.02	1.01	0.996	0.105	1.01	1.00
20	0.993	0.279	1.03	1.00	0.999	0.084	1.01	1.00
15	0.981	0.339	1.05	1.00	0.985	0.290	1.00	1.00
10	0.901	0.214	1.05	1.00	NF[c]	NF	NF	NF
7	0.951	0.176	1.05	1.00	0.976	0.119	1.03	1.00
4	0.918	0.203	1.04	1.00	0.959	0.137	1.03	1.00

[a] The statistical indexes were obtained by fitting the growth or survival data of *V. parahaemolyticus* on shrimp during storage after AEW treatment.
[b] The statistical indexes were obtained by fitting the growth or survival data of *V. parahaemolyticus* on shrimp during storage after sterile 0.85% saline solution treatment.
[c] NF means that the experimental data could not be fitted by the models, because the fit produced unacceptable values of R^2.

2.7. Statistical analysis

The following statistical indicators were used to compare the performance of the models: correlation coefficients (R^2), the p-values from the Fisher F-test; root mean square error (RMSE, Eq. (3)), accuracy factor (A_f, Eq. (4)), bias factor (B_f, Eq. (5)).

$$RSME = \sqrt{\frac{\sum (obs - pred)^2}{n}} \quad (3)$$

$$A_f = 10^{\left(\frac{\sum |Log(pred/obs)|}{n}\right)} \quad (4)$$

$$B_f = 10^{\left(\frac{\sum Log(pred/obs)}{n}\right)} \quad (5)$$

where *obs* is observed values, *pred* is predicted values by models, and the n stands for the number of observations. The RMSE values approaching zero indicate a closer fit with the data for the model (Huang et al., 2014). A_f provides the accuracy of the model, which reflects how close the predicted values are to the observed values, while B_f indicates the mean difference between observed and predicted value (Ross, 1996).

The modified Gompertz model and Log-linear model were fitted using Origin pro 8.0 (Origin Lab Corp., Northampton, USA). Obtained values were expressed as the means ± standard deviation. Statistical analysis was performed using SPSS statistical package 17.0 (SPSS Inc., Chicago, IL). Values differences were compared using the least significant difference (*LSD*) method at $p = 0.05$.

3. Results and discussion

3.1. Performance of models

Statistical indexes for the modified Gompertz model and Log-linear model under each temperature are presented in Table 1. The models had a high degree of goodness-of-fit quantified by R^2 with its values ranging from 0.901 to 0.999 and a statistical significance level of $p < 0.01$ from the F-test. Moreover, RMSE values ranged from 0.084 to 0.344 further demonstrating a higher degree of correlation between experimental and fitted values calculated from models (Huang et al., 2014).

Additionally, internal evaluations based on the data to build the models were applied to evaluate the performance of models as described by McClure et al. (1997). The range of A_f and B_f values calculated for the models was 1.0–1.06 and 1.0–1.01, respectively. All these values were within the acceptable limit according to the principles suggested by Ross et al. (2000). Therefore, the modified Gompertz model and Log-linear model had a good statistical performance and could be used to describe the experimental data adequately.

3.2. Changes on growth of V. parahaemolyticus on shrimp during post-AEW storage

Fig. 1A–D illustrates the growth curves of *V. parahaemolyticus* on shrimp treated with AEW stored at different temperatures (15 °C, 20 °C, 25 °C, 30 °C). It could be observed that AEW treatment obviously affected the growth behavior of *V. parahaemolyticus* on shrimp during storage, resulting in a right-shift on the growth curves of *V. parahaemolyticus* compared with control.

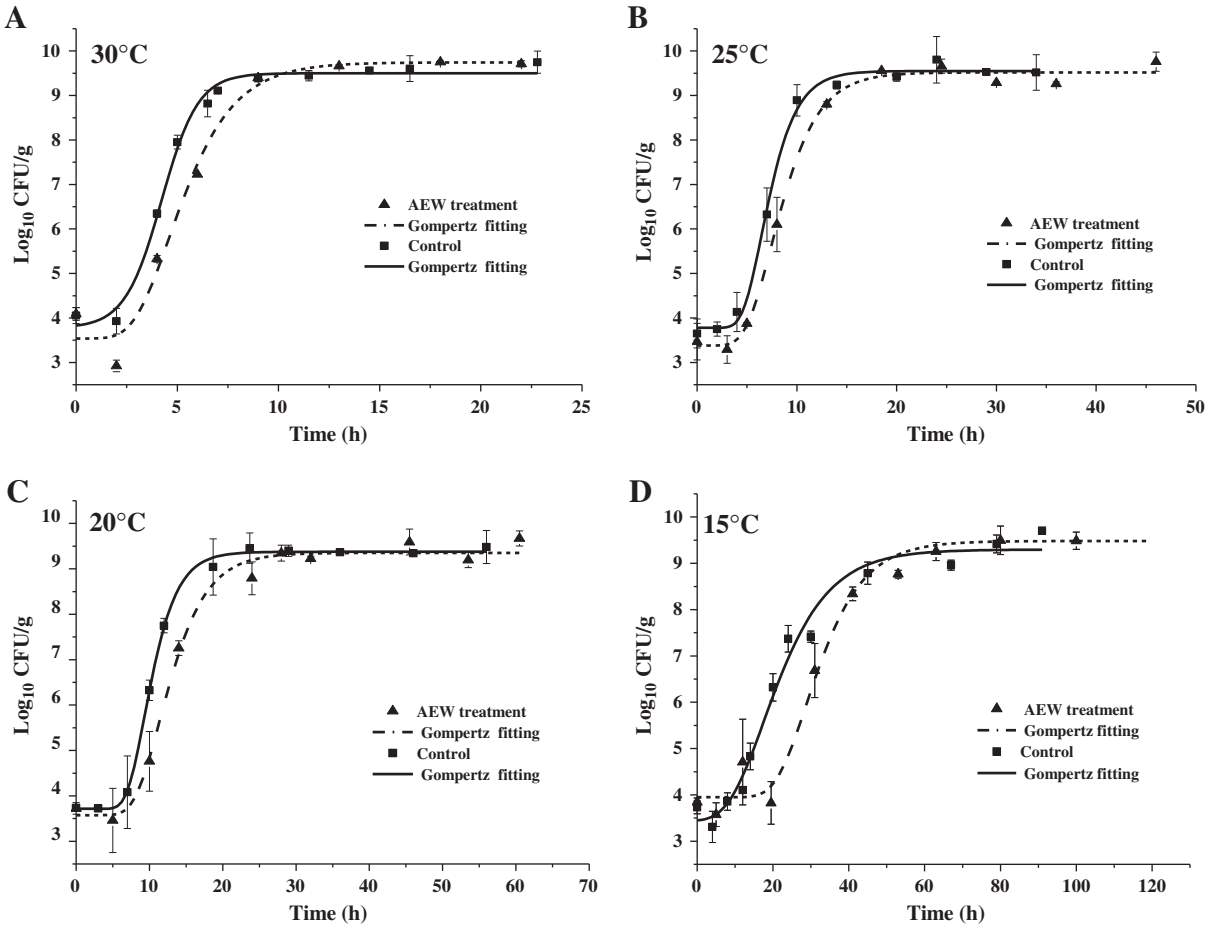

Fig. 1. Growth curves of *V. parahaemolyticus* on shrimp treated and untreated (control) by acidic electrolyzed water (AEW) stored at 30 °C (A), 25 °C (B), 20 °C (C), and 15 °C (D) fitted by modified Gompertz model using plate count enumeration. Two trials with two replicates per trial were done for each temperature. Error bars represent standard deviations.

The obtained growth kinetic parameters fitted by the modified Gompertz model are presented in Table 2. The results showed that μ_{max} of *V. parahaemolyticus* had values ranging from 0.25 to 1.28 Log_{10} CFU g^{-1} h^{-1} in treated samples, and ranging from 0.23 to 1.60 Log_{10} CFU g^{-1} h^{-1} in control. Meanwhile, μ_{max} had a positive relevance with the storage temperature, and the correlation coefficient could reach 0.997 for AEW treatment and 0.989 for control. Statistically, there was significant difference ($p \leq 0.016$) in μ_{max} of *V. parahaemolyticus*, regardless of shrimp samples treated with AEW or not, under different storage temperatures. The above analysis indicated that temperature played an important role in affecting the growth behavior of *V. parahaemolyticus* on shrimp, which has also been found in study done by Boonyawantang et al. (2012). However, AEW treatment presented a stronger capability to suppress the growth of *V. parahaemolyticus* on shrimp, because the values of μ_{max} was always lower ($p \leq 0.031$) than control in combination with storage temperatures, except at 15 °C.

Additionally, in Table 2 the lag phase (λ) of *V. parahaemolyticus* on shrimp was prolonged with the storage temperature decreasing, ranging from 2.54 h to 8.46 h in control samples. However, AEW treatment possessed a markedly ($p < 0.05$) effect to prolong the lag phase (λ) of *V. parahaemolyticus*, with the values ranging from 2.83 h to 21.71 h. The maximum ratio of lag phase (λ) of *V. parahaemolyticus* on shrimp could be reached at 2.6 times at 15 °C between AEW treatment and control. Statistically, the lag phase of *V. parahaemolyticus* was significantly ($p \leq 0.027$) longer than control at 20 °C and 15 °C, while no significant difference was observed when stored at 30 °C and 25 °C after AEW treatment. Similar trends have been found in the study done by Ding et al. (2010). Nevertheless, AEW treatment could not obviously ($p > 0.05$) decrease the maximum population density with the storage time adequate.

Table 2
Growth kinetic parameters of *V. parahaemolyticus* on shrimp treated and untreated (Control) with acidic electrolyzed water (AEW) fitted by modified Gompertz model stored at different temperatures.

Temperature (°C)	μ_{max} (Log_{10} CFU/g/h)[e]		λ (h)[e]		D (Log_{10} CFU/g)[e]	
	AEW	Control	AEW	Control	AEW	Control
30	1.28 ± 0.03^{aB}	1.60 ± 0.13^{aA}	2.83 ± 0.04^{dA}	2.54 ± 0.18^{dA}	9.74 ± 0.02^{aA}	9.55 ± 0.03^{aA}
25	0.87 ± 0.02^{bB}	1.11 ± 0.16^{bA}	4.86 ± 0.35^{cA}	4.57 ± 0.68^{cA}	9.52 ± 0.02^{aA}	9.55 ± 0.09^{aA}
20	0.61 ± 0.05^{cB}	0.85 ± 0.01^{bA}	7.77 ± 0.43^{bA}	6.92 ± 0.01^{bB}	9.35 ± 0.08^{aA}	9.38 ± 0.02^{aA}
15	0.25 ± 0.04^{dA}	0.23 ± 0.00^{cA}	21.71 ± 1.23^{aA}	8.46 ± 0.59^{aB}	9.48 ± 0.13^{aA}	9.30 ± 0.07^{aA}

[a–d]Mean values followed by different letters in the same column differ significantly by *LSD* test ($p \leq 0.05$);
[A–B]Mean values followed by different letters in the same row differ significantly by *LSD* test ($p \leq 0.05$).
[e]Means ± standard deviation were calculated based on the data from two trials with two replicates per trial at different temperatures.

In this work, AEW-treated *V. parahaemolyticus* showed a greater extension of lag time and slower growth rate compared with control, which can be mainly attributed to the occurrence of sub-lethal damage by AEW based on the analysis in the studies by Silva-Angulo et al. (2014) and Moosekian et al. (2014). Because sub-lethal cells as a consequence of mild treatments need more time to recover and adapt before they have the ability to multiply. Additionally, it has been reported that the duration of the lag phase of several food borne microorganism depends inversely on the inoculum size in culture media under a restricted or stressed range of conditions (Augustin et al., 2000; Kaprelyants and Kell, 1996; Pascual et al., 2001; Robinson et al., 2001; Silva-Angulo et al., 2014). However, it was generally assumed that the bacterial growth rate was independent of the inoculum size (Silva-Angulo et al., 2014). In food products, the contaminated populations of cells were lower than those survived from AEW treatment on shrimp in this study (3.6 Log_{10} CFU/g). Therefore, AEW treatment could help the seafood industry to obtain longer lag phase of survival microorganisms prior to growth in combination with suitable storage temperature, thereby reducing the risk of illness due to the proliferation of pathogens.

3.3. Changes on inactivation of *V. parahaemolyticus* on shrimp during post-AEW storage

Fig. 2A–C describes the survival of *V. parahaemolyticus* on shrimp under different temperatures (10 °C, 7 °C, 4 °C) after AEW treatment. Overall, the number of *V. parahaemolyticus* on shrimp could be reduced when the temperature was below 10 °C, regardless of samples treated with AEW or not. These results were identical to previous reports indicating that *V. parahaemolyticus* was sensitive and gradually inactivated at refrigeration temperature (Lin et al., 2004; Yang et al., 2009; Yoon et al., 2008). However, the present study demonstrated that AEW treatment could significantly ($p < 0.05$) enhance the inactivation rate of *V. parahaemolyticus* after post-treatment storage.

In Fig. 2A, a rapid drop in bacterial counts on shrimp appeared during the initial storage when the temperature was 10 °C, especially in AEW-treated samples. Based on Log-linear model, AEW-treated samples showed an average inactivation value K of 2.75×10^{-2} Log_{10} CFU/g/h with the initial loads of *V. parahaemolyticus* at 4.69 Log_{10} CFU/g (Table 3) during the whole storage period. However, this phenomenon was not observed in control samples. For control samples, there was an increase in the populations of *V. parahaemolyticus* after 85 h storage and the populations exceeded the initial inoculation reaching 6.04 Log_{10} CFU/g at 196 h, although the *V. parahaemolyticus* showed an initial rapid drop within 85 h. To the best of our knowledge, similar findings have not been reported in previous studies. This evidence of increment of pathogenic numbers in untreated samples supports the guidelines of chilling preservation in which a safe temperature zone should be at lower than 8 °C (U.S. Food and Drug Administration, 1997). Thus, these results suggested that AEW treatment could greatly reduce the potential risk of illness caused by *V. parahaemolyticus* on shrimp through inactivating pathogens during post-treatment storage at 10 °C.

The survival of AEW-treated *V. parahaemolyticus* on shrimp stored at 7 °C and 4 °C are shown in Fig. 2B–C and Table 3, respectively. It could be obviously observed that a relatively rapid decrease in populations of *V. parahaemolyticus* on shrimp occurred in AEW-treated samples. Furthermore, the inactivation value K (6.85×10^{-2} and 6.52×10^{-2} Log_{10} CFU/g/h) of *V. parahaemolyticus* stored at 7 °C and 4 °C

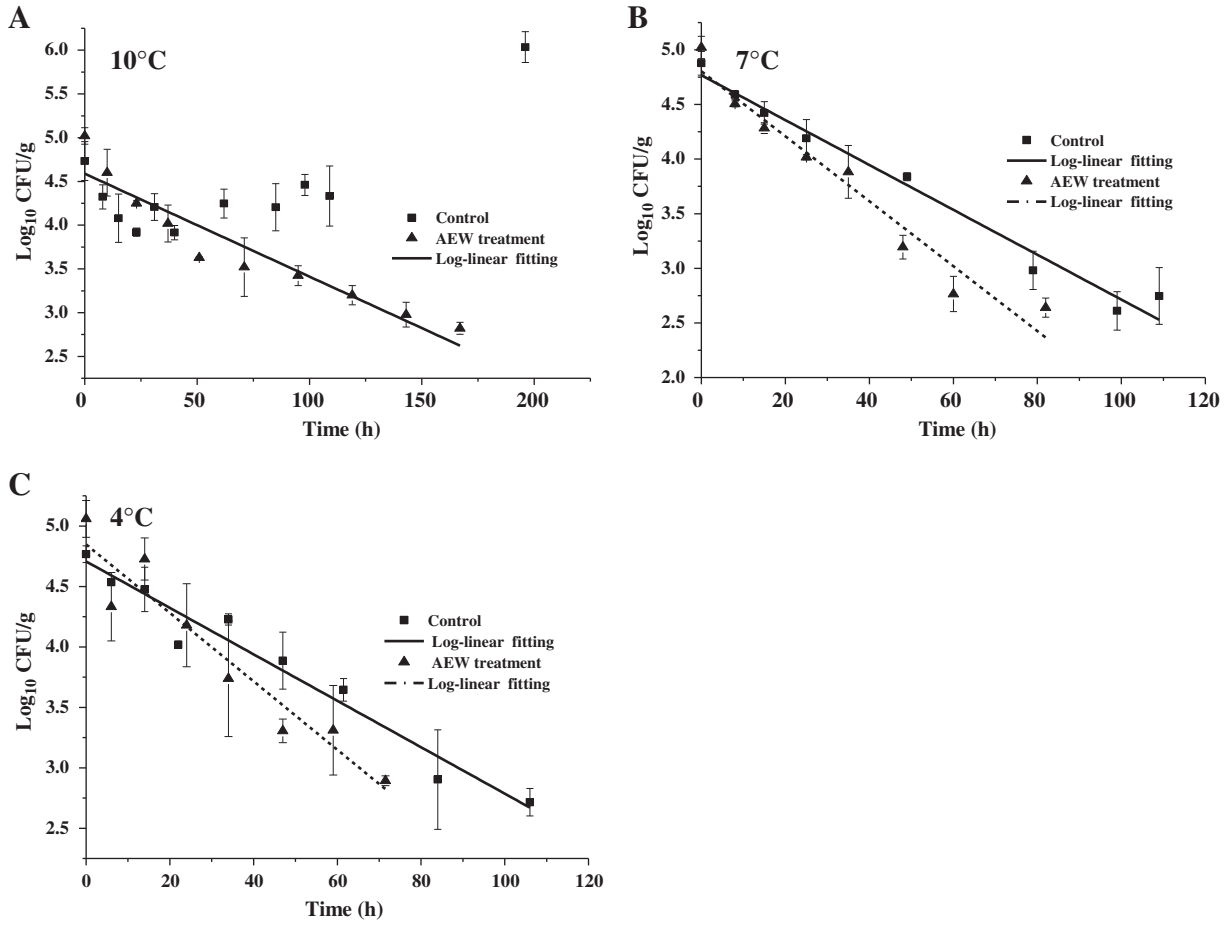

Fig. 2. Survival curves of *V. parahaemolyticus* on shrimp treated and untreated (control) by acidic electrolyzed water (AEW) stored at 10 °C (A), 7 °C (B), and 4 °C (C) fitted by log-linear model using plate count enumeration. Two trials with two replicates per trial were done for each temperature. Error bars represent standard deviations.

Table 3
Survival kinetic parameters of *V. parahaemolyticus* on shrimp treated and untreated (control) with acidic electrolyzed water (AEW) fitted by Log-linear model stored at refrigeration temperatures.

Temperature (°C)	$Log(S_0)$ (Log_{10} CFU/g)[e]		$\|K\| \times 10^{-2}$ (Log_{10} CFU/g/h)[e]	
	AEW	Control	AEW	Control
10	4.69 ± 0.13[a]	NF[c]	2.75 ± 0.07[b]	NF[c]
7	4.80 ± 0.03[aA]	4.77 ± 0.03[aA]	6.85 ± 0.10[aA]	4.73 ± 0.04[aB]
4	4.85 ± 0.04[aA]	4.71 ± 0.03[aA]	6.52 ± 0.32[aA]	4.42 ± 0.23[aB]

[a–b]Mean values followed by different letters in the same column differ significantly by *LSD* test ($p \leq 0.05$).
[A–B]Mean values followed by different letters in the same row differ significantly by *LSD* test ($p \leq 0.05$).
[c]NF means that the experimental data could not be fitted by the models, because the fit produced unacceptable values of R^2.
[e]Means ± standard deviation were calculated based on the data from two trials with two replicates per trial at different temperatures.

were markedly higher than that (2.75×10^{-2} Log_{10} CFU/g/h) at 10 °C. However, there was no significant difference in the inactivation value *K* between 7 °C and 4 °C. Compared with control, AEW treatment presented higher ($p < 0.05$) inactivation value *K*, indicating that AEW treatment increased the sensitivity of *V. parahaemolyticus* to refrigeration temperatures.

Bacterial inactivation by AEW is well documented with oxidative ability of available chlorine concentration (ACC, including HOCl, OCl^-, Cl_2) directed towards the cell membrane, various metabolic functions, etc. (Huang et al., 2008; Moosekian et al., 2014). Under this condition, a high ratio of injured cells could be induced by ACC as above mentioned (Moosekian et al., 2014). Moreover, the refrigeration temperature could gradually inactivate *V. parahaemolyticus* (Lin et al., 2004; Yang et al., 2009; Yoon et al., 2008). Therefore, stored at unfavorable temperatures (<10 °C), the injured cells of *V. parahaemolyticus* on shrimp could not undergo cellular repair, resulting in rapid reduction of the populations of this pathogen in comparison to control samples in Fig. 2 using plate count enumeration.

3.4. Fate of *V. parahaemolyticus* on shrimp during post-AEW storage reveled by real-time PCR

In order to better understand the fate of *V. parahaemolyticus* on shrimp stored at refrigeration temperatures, real-time PCR was used to study the population dynamics of this pathogen from the viewpoint of DNA quantification, due to its high specificity and sensitivity (Nogva et al., 2000; Ye et al., 2012).

As displayed in Fig. 3A, each experimental data point of AEW-treated *V. parahaemolyticus* populations quantified by real-time PCR was very close to the line of average value when the samples were stored at 10 °C for 106 h, which suggested that the amount of DNA of *V. parahaemolyticus* on shrimp remained constant after AEW treatment. Thus, the results indicated that the physiological state of AEW-treated *V. parahaemolyticus* did not involve in the phase of cells growth, indicating that AEW treatment completely suppressed the proliferation of cells during post-treatment storage comparing with control. Whereas, significant increase in the populations of *V. parahaemolyticus* ranging from 5.38 to 8.16 Log_{10} CFU/g was observed in control samples by real-time PCR in Fig. 3A, and the growth curve could be fitted by the modified Gompertz model, suggesting that the increase of cells followed the sigmoidal bacterial growth. While, the fate of *V. parahaemolyticus* revealed by real-time PCR was not identical with those presented in control samples by microbial counts in Fig. 2A, showing a rapid decline in the populations of *V. parahaemolyticus* during initial 85 h storage. Therefore, the present study revealed that *V. parahaemolyticus* on shrimp without treatment still had the ability to perform the multiplication of cells at 10 °C, although a rapid decrease in counts appeared. This situation actually results in a true risk of rapidly reaching the bacterial concentration necessary for causing illness (6 Log_{10} CFU) (U.S. Food and Drug Administration, 2005), once shrimp are exposed to temperature-abused environment before consumption. However, AEW treatment could greatly reduce the risk of seafood safety on basis of the above analysis.

Real-time PCR was employed to describe the fate of *V. parahaemolyticus* on control samples stored at 7 °C and 4 °C, sequentially (Fig. 2B). The proliferation of *V. parahaemolyticus* was completely inhibited. Furthermore, the experimental data could be fitted by Log-linear model with the values of *K* approaching zero, indicating that the populations of *V. parahaemolyticus* on shrimp were almost kept constant as showed in AEW treated samples in Fig. 3A. Thus, this study also became the first one to reveal the fate of *V. parahaemolyticus* on shrimp after AEW treatment using real-time PCR and microbial counts, comprehensively.

4. Conclusion

Besides its stronger bactericidal activity, AEW treatment can greatly prolong the lag phase and decrease the specific growth rate of

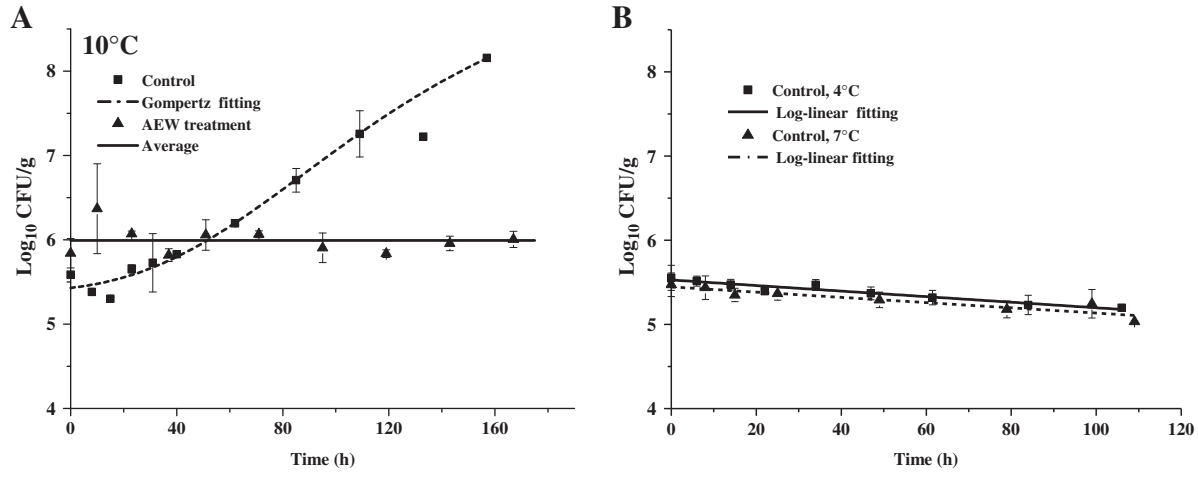

Fig. 3. Growth and survival curves of *V. parahaemolyticus* on shrimp treated and untreated (control) by acidic electrolyzed water (AEW) stored at refrigeration temperatures obtained by real-time PCR. (A) The populations of *V. parahaemolyticus* on shrimp treated and untreated with AEW stored at 10 °C quantified by real-time PCR; dash-dot line represents the modified Gompertz model; solid line represents average value of experimental data from AEW-treated samples; (B) the populations of *V. parahaemolyticus* on shrimp untreated with AEW stored at 7 °C and 4 °C quantified by real-time PCR; dash-dot line and solid line represent log-linear model. Two trials with two replicates per trial were done for each temperature. Error bars represent standard deviations.

V. parahaemolyticus on shrimp during the post-treatment storage, while it can accelerate the inactivation of *V. parahaemolyticus* when AEW-treated shrimp were stored at refrigeration temperatures. In addition, the experimental data from real-time PCR revealed that AEW treatment can completely suppress the multiplication of cells of *V. parahaemolyticus* on shrimp, especially for 10 °C storage. Based on above analysis, this study provided more comprehensive information to understand the fate of *V. parahaemolyticus* on seafood treated with AEW, hence to pave the way for reducing the risk of illness caused by *V. parahaemolyticus* by taking efficient measures.

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