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A predictive model for assessment of decontamination effects of lactic acid and chitosan used in combination on *Vibrio parahaemolyticus* in shrimps

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ABSTRACT

Vibrio parahaemolyticus is a major causative agent of human gastroenteritis in seafood products including shrimps. Lactic acid and chitosan are natural antimicrobials for food decontamination in the washing process of seafood. In this research, a 4-factor response surface model based on the Box–Behnken experimental design was developed to evaluate the effects of lactic acid (1%, 2%, and 3%, v/v), chitosan (0.4%, 1%, and 1.6%, w/v), rotational rate (90, 110, and 130 rpm) and washing time (10, 20, and 30 min) on reduction of *V. parahaemolyticus* inoculated in raw shrimps. These treatments achieved 2.2 to 4.3 $\log_{10}$ CFU/g reduction of *V. parahaemolyticus* in shrimps. Stepwise stratification led to a simplified model that has a satisfactory performance as evidenced by statistical indices ($R^2 = 0.92$; $p < 0.0001$; RMSE = 0.196) and external validation parameters [bias factor (B_f) = 1.01; accuracy factor (A_f) = 1.05]. The model generated an optimum treatment combination (3% lactic acid, 1.6% chitosan, and rotational rate at 110 rpm) that could achieve greatest bacterial reduction of 4.5 $\log_{10}$ CFU/g. Among the four factors, lactic acid and chitosan were the major contributors for bacterial decontamination. Analysis of variances showed a significant interactive inactivation effect ($p < 0.05$) from combined use of lactic acid and chitosan. The treatments did not have adverse effects on the quality attributes such as color and pH of the shrimps.

1. Introduction

Vibrio parahaemolyticus, a halophilic Gram-negative rod-shaped bacterium widely distributed in marine environments, has been recognized as one of the leading causes of foodborne outbreaks associated with seafood consumption around the world (Baumann and Schubert, 1984; Iwahori and Yamamoto, 2010). In recent years, consumer demand for shrimp has steadily increased throughout the world (Norhana et al., 2010). High prevalence of *V. parahaemolyticus* in shrimps has been reported in many countries, especially in Asia, including Thailand, Iran and China (Minami et al., 2010; Zarei et al., 2012; Zhang et al., 2007). A number of human gastroenteritis outbreaks have been reported associated with the consumption of raw or undercooked shrimp contaminated with *V. parahaemolyticus*, posing potential risk to public health and indicating the need to mitigate bacterial load in shrimps (Cabanillas-Beltrán et al., 2006; CSPI, 2007; Yang et al., 2008).

A variety of intervention methods have been investigated for eliminating pathogens in shrimps. Intervention methods, such as thermal treatment, high pressure and irradiation, have been reported to be alternatives to inactivate *V. parahaemolyticus* in seafood (Andrews et al., 2000; Ma and Su, 2011; Mahmoud, 2009). However, treatments with high temperature (>52.5 °C) or pressure (>800 MPa) could lead to undesirable changes in color, flavor and/or texture (Andrews et al., 2003; Romero et al., 2004). In order to minimize the adverse effects on nutrition and sensory characteristics of food, there is a strong need to develop effective and safe intervention methods in post-harvest shrimp processing (Norhana et al., 2010).

Lactic acid has been generally recognized as safe (GRAS) under 21CFR 184.1061 by the U.S. Food and Drug Administration (FDA, 2011b) and shown as a potential disinfectant against pathogenic bacteria on beef, vegetables and fruits (El-Khateib et al., 1993; Lin et al., 2002; Venkitanarayanan et al., 2002). Treatment with 1–3% lactic acid for 10–15 min could reduce *V. parahaemolyticus* by 2–3 $\log_{10}$ CFU/g without adverse effects on sensory properties in seafood (Shirazinejad et al., 2010; Terzi and Gucukoglu, 2010). Chitosan, rich in shells of crustaceans, such as crabs and shrimps, was initially used as a food preservative by coating the food surface against spoilage microorganisms (No et al., 2007). With regard to the antimicrobial effects of chitosan in seafood products, several studies revealed limited effect of chitosan on bacterial inactivation (Lopez-Caballero et al., 2005; Ye et al., 2008), while some other studies reported its effective antibacterial activities against a variety of pathogenic bacteria (Cao et al., 2009; Roller and Corvill, 2000; No et al., 2007; Terzi and Gucukoglu, 2010). The reason

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for the difference could be that the inactivation activity of chitosan depends on its molecular weight, degree of deacetylation, bacterial strains, and food matrix (Alishanhi and Aïder, 2012; Devlieghere et al., 2004; Kong et al., 2010). Alishanhi and Aïder (2012) reviewed the applications of chitosan in seafood industry and suggested that it could be successfully incorporated into seafood products to improve seafood safety.

Predictive microbiology has been used as an important tool to improve food safety by developing mathematical models to quantitatively predict the growth and survival of microorganisms under prescribed environmental conditions during food processing and storage (McMeekin et al., 1997; Whiting and Buchanan, 1994; Yang et al., 2009; Li et al., 2011). In recent years, response surface models have been used for optimization of treatment conditions for prediction of pathogen inactivation in fresh-cut vegetables, fruits and meat. The model can be used to analyze individual or combined effects of independent factors in the experiments (Bas and Boyaci, 2007; Kwak et al., 2011; Lahlali et al., 2008; Bover-Cid et al., 2012). Here, we report a response surface model for prediction of *V. parahaemolyticus* survival in shrimps subjected to combined washing with lactic acid and chitosan. Changes of color and pH of treated shrimps were also examined.

2. Materials and methods

2.1. Bacterial strains and chemicals

Two strains of *V. parahaemolyticus* used in this study were ATCC 33847 (ATCC, USA) and KP9 (a clinical strain from a patient). The bacteria were stored at −80 °C in brain heart infusion broth (BHI; BD, Sparks, MD, USA) containing 20% glycerol. Each strain was separately incubated in tryptone soya broth (TSB; BD, Sparks, MD, USA) supplemented with 3% (w/v) sodium chloride (NaCl, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at 37 °C for 12 h, and subcultured in TSB-3% NaCl for 6 h (approximately 10^8 CFU/ml). Lactic acid was from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, and chitosan (MW = 20 kDa and 98% degree of deacetylation) was purchased from Golden-Shell Biochemical Co., Ltd., Yuhuan, Zhejiang, China.

2.2. Shrimps and inoculation

Fresh shrimps were purchased from a local market one to two days before each experiment. The shrimps were beheaded and stored in a freezer at −20 °C. They were thawed overnight at 4 °C the day before use, peeled, immersed in 75% ethanol for 2 min and rinsed with sterile Milli-Q water (Millipore, Billerica, MA, USA) for 3 times to eliminate residual ethanol. Shrimp samples (6 ± 0.3 g per shrimp) were then submerged into 200 ml of *V. parahaemolyticus* suspensions (about 10^8 CFU/ml) for 30 min and transferred to plastic plates for another 30 min to allow bacterial attachment, both at room temperature. The inoculated shrimps were used for the subsequent treatments.

2.3. Experimental design

The experiment was designed based on our preliminary experiments and processing conditions mimicking the processing practices. In the present study, the concentrations of lactic acid and chitosan were considered as the main factors to affect survival of *V. parahaemolyticus* in shrimps. To achieve desirable bacterial reduction and avoid adverse changes of color and pH, the level of lactic acid was designated at 1–3% and the concentration of chitosan should be more than 0.4% based on our preliminary results and previous studies (Shirazinejad et al., 2010; Terzi and Gucukoglu, 2010). Considering that the solution could become very viscous at concentrations higher than 1.6% and not suitable for industrial application, the level of chitosan was selected within 0.4–1.6%. Other factors considered to affect bacterial survival included treatment time and rate of rotation. Rotation represents a factory situation where shrimps could be hand-shaken in a bucket for a few minutes. Based on the improvement of bacterial inactivation and cost of time and energy in seafood processing, the rotational rate and treatment time were selected within 90–130 rpm and 10–30 min, respectively. To stratify the effects of these four factors, response surface methodology based on the Box-Behnken experimental design was used by incorporating different levels of each factor: lactic acid (X_1: 1%, 2% and 3%) (v/v), chitosan (X_2: 0.4%, 1.0% and 1.6%) (w/v), rotational rate (X_3: 90, 110 and 130 rpm) and washing time (X_4: 10, 20 and 30 min). Three levels for each factor were coded as "−1, 0, and +1" according to Eq. 1 (Gao and Ju, 2007) in Table 1, with a total of 27 runs according to the experimental design (Table 2).

$$x_i = (X_i - X_0)/\Delta X \quad (1)$$

where x_i is the coded value of the variable X_i, X_0 is the value of X_i at central point, and ΔX is the step change.

2.4. Inactivation treatments

Lactic acid and chitosan were dissolved in sterile distilled water (supplemented with 3% NaCl) with different concentration combinations as the antimicrobial solutions. Inoculated shrimp samples were treated with 100 ml of corresponding antimicrobial solutions in sterile beakers which were then placed in a shaker set at 90, 110 and 130 rpm for 10, 20 or 30 min at room temperature (25 ± 2 °C). At the end of treatment, the shrimp samples were transferred to individual sterile bags containing 20 ml of buffered peptone water (BPW, BD, Sparks, MD, USA) containing 3% NaCl and homogenated for 2 min in a food stomacher (Model 400, Seward, London, UK) for bacterial counting. Each treatment consisted of three replicates. For each trial, three inoculated shrimps were treated with 3% sterile saline as positive controls to enumerate the initial inoculation load.

2.5. Bacterial enumeration

Survival of *V. parahaemolyticus* to treatments was examined by the spiral plating method. The homogenates were serially diluted 10-fold in BPW plus 3% NaCl and appropriate dilutions were automatically spread on the thiosulfate citrate bile salts sucrose agar (TCBS; BD, Sparks, MD, USA) in duplicate with a spiral plater (WASP 2, Don Whitley Scientific, Shipley, UK). The plates were incubated at 37 °C for 16–18 h. Colonies on the TCBS agar plates were enumerated by a ProtoCOL automated colony counter (Synbiosis, Cambridge, UK), and the limit of detection was one colony for 50 μl sample (20 CFU/ml). Each datum point of bacterial survival represents an average of two individual experiments with each treatment containing triplicate samples.

2.6. Model development and validation

A survival model was developed with the JMP10 software (SAS Institute, Cary, NC). The response was measured as population reduction of *V. parahaemolyticus* in shrimps. A quadratic polynomial model as shown

Table 1
Levels of variables in the experiments.

Factor	Symbol[a]		Level		
	Coded	Uncoded	−1	0	+1
Lactic acid (%, v/v)	x_1	X_1	1	2	3
Chitosan (%, w/v)	x_2	X_2	0.4	1.0	1.6
Rotational rate (rpm)	x_3	X_3	90	110	130
Time (min)	x_4	X_4	10	20	30

[a] $x_1 = (X_1 - 2)/1$; $x_2 = (X_2 - 1.0)/0.6$; $x_3 = (X_3 - 110)/20$; $x_4 = (X_4 - 20)/10$.

Table 2
Experimental design and population reduction of *Vibrio parahaemolyticus* in shrimps.

Run	Variables				Reduction[a] ($\log_{10}$ CFU/g)
	Lactic acid (%, v/v)	Chitosan (%, w/v)	Rotational rate (rpm)	Time (min)	
1	2	1.6	90	20	3.12 ± 0.34^{efgh}
2	1	1	90	20	2.17 ± 0.36^{l}
3	2	1	90	10	2.61 ± 0.43^{ghijkl}
4	3	1	90	20	3.51 ± 0.40^{cde}
5	2	0.4	90	20	2.44 ± 0.43^{hijkl}
6	2	1	90	30	2.95 ± 0.30^{efghijk}
7	3	1	110	30	3.98 ± 0.42^{abcd}
8	1	0.4	110	20	2.25 ± 0.33^{kl}
9	3	1.6	110	20	4.20 ± 0.68^{ab}
10	2	1.6	110	10	3.30 ± 0.24^{efg}
11	2	0.4	110	30	3.02 ± 0.37^{efghij}
12	3	0.4	110	20	3.12 ± 0.20^{efgh}
13	2	1.6	110	30	3.45 ± 0.31^{def}
14	1	1	110	10	2.40 ± 0.40^{ijkl}
15	2	1	110	20	3.11 ± 0.29^{efgh}
16	2	1	110	20	3.02 ± 0.20^{efghi}
17	1	1	110	30	2.61 ± 0.29^{ghijkl}
18	3	1	110	10	4.32 ± 0.41^{a}
19	2	1	110	20	3.15 ± 0.11^{efgh}
20	2	0.4	110	10	2.66 ± 0.51^{ghijkl}
21	1	1.6	110	20	2.38 ± 0.50^{hijkl}
22	2	1.6	130	20	3.57 ± 0.46^{bcde}
23	1	1	130	20	2.35 ± 0.32^{jkl}
24	2	1	130	30	3.12 ± 0.27^{efgh}
25	2	1	130	10	2.75 ± 0.25^{fghijkl}
26	3	1	130	20	4.14 ± 0.42^{abc}
27	2	0.4	130	20	2.59 ± 0.41^{ghijkl}

[a–l] Mean values followed by different superscript letters are significantly different ($p < 0.05$).
[a] Values are means ± standard deviations (n = 6). The initial inoculation load of *Vibrio parahaemolyticus* in shrimps determined by positive controls was 7.2 ± 0.3 $\log_{10}$ CFU/g.

in Eq. 2 was employed to predict the reduction of *V. parahaemolyticus* (Y) in shrimps subjected to washing with lactic acid and chitosan:

$$Y = k_0 + k_1x_1 + k_2x_2 + k_3x_3 + k_4x_4 + k_5x_1^2 + k_6x_2^2 + k_7x_3^2 + k_8x_4^2 + k_9x_1x_2 + k_{10}x_1x_3 + k_{11}x_1x_4 + k_{12}x_2x_3 + k_{13}x_2x_4 + k_{14}x_3x_4 \quad (2)$$

where, Y is the bacterial reduction ($\log_{10}$ CFU/g); x_1, x_2, x_3 and x_4 represent coded variables, including lactic acid, chitosan, rotational rate, and washing time, respectively. k_0 is the constant; k_1, k_2, k_3 and k_4 are linear coefficients; k_5, k_6, k_7 and k_8 are quadratic coefficients; k_9, k_{10}, k_{11}, k_{12}, k_{13} and k_{14} are interaction coefficients. Statistical analysis of variance (ANOVA) was used to evaluate significance and adequacy of the model. Only significant variables based on the F-test results were selected by a stepwise regression to simplify the model.

To validate the model, eight additional trials with random combinations of four variables were conducted (Table 3). The method of bacterial inactivation in shrimps was the same as explained previously for the inactivation treatments. The selected parameters were within the original range of the experimental design but not included in the establishment of the model. The observed results were used to evaluate performance of the model by the bias factor (B_f) (Eq. (3)) and the accuracy factor (A_f) (Eq. (4)) proposed by Ross (1996).

Table 3
Eight independent trials to validate the predictive model for inactivation of *Vibrio parahaemolyticus* in shrimps.

Run	Lactic acid (%, v/v)	Chitosan (%, w/v)	Rotational rate (rpm)	Bacterial reduction ($\log_{10}$ CFU/g)	
				Observed	Predicted
1	2.5	1.2	100	3.05 ± 0.26	3.34
2	1.5	0.8	100	2.31 ± 0.30	2.36
3	2.5	0.8	100	3.07 ± 0.13	3.04
4	1.5	1.2	100	2.70 ± 0.19	2.50
5	1.5	1.2	120	2.62 ± 0.39	2.54
6	2.5	0.8	120	3.27 ± 0.32	3.08
7	1.5	0.8	120	2.58 ± 0.18	2.40
8	2.5	1.2	120	3.30 ± 0.46	3.38

$$B_f = 10^{\left[\sum_{i=1}^{n} \log\left(\frac{obs}{pred}\right)/n\right]} \quad (3)$$

$$A_f = 10^{\left[\sum_{i=1}^{n} \left|\log\left(\frac{obs}{pred}\right)\right|/n\right]} \quad (4)$$

where n is the number of trials, *obs* is the observed values of bacterial reduction ($\log_{10}$ CFU/g), and *pred* is the predicted values of bacterial reduction ($\log_{10}$ CFU/g).

2.7. Measurement of color and pH of the shrimp samples

Color changes of the treated shrimps were determined by placing the probe of the colorimeter Chroma Meter CR 400 (Minolta Osaka, Japan) onto the shrimp surface. Values of L, a, and b representing lightness, redness and yellowness, respectively, were recorded. The pH values of treatment solutions were measured with a Mettler Toledo pH meter (Model FE20; Toledo Instruments Co. Ltd., Switzerland), and those of shrimps were measured with a portable pH meter (Hach H160, Loveland, CO, USA) by directly inserting the probe into the shrimps. All measurements were taken on three sites of each treated sample.

3. Results

3.1. Reduction of V. parahaemolyticus in shrimps

The initial inoculation load in shrimps determined by positive controls was approximately 7.2 $\log_{10}$ CFU/g. Microbial reduction for all combined treatments of lactic acid and chitosan, treatment time and rotational rate are summarized in Table 2. Washing with the antimicrobial solutions achieved significant reduction of *V. parahaemolyticus* in shrimps, ranging from 2.2 to 4.3 $\log_{10}$ CFU/g. Among the four variables, lactic acid was the most significant to decontaminate the bacteria ($p < 0.0001$). Washing with 3% lactic acid combined with any other factors could lead to reduction by more than 3 $\log_{10}$ CFU/g.

3.2. Response surface modeling of V. parahaemolyticus inactivation

A backward stepwise regression was carried out using JMP software to develop a simplified response surface model only with the significant variables. The lack of fit test was conducted to evaluate the systematic variations unaccounted for in the hypothesized model (a significant lack of fit indicates that the model does not describe the data well). By using ANOVA on the estimated parameters of all variables, we found statistical significance with three linear coefficients (x_1, x_2 and x_3) ($p < 0.0001$), one interaction coefficient ($x_1 \cdot x_2$) ($p < 0.05$), and the quadratic coefficient (x_3^2) ($p < 0.05$) for estimation of bacterial reduction to the treatments. A summary of the estimated parameters for coded variables with significances is given in Table 4. The simplified model is shown as Eq. 5. The model has a good statistical performance as shown by R^2 (0.92), probability value ($p < 0.0001$) and the lack of fit test ($p > 0.05$).

$$Y = -4.66 + 0.36X_1 - 0.25X_2 + 0.11X_3 + 0.40X_1X_2 - 0.00047{X_3}^2 \quad (5)$$

Table 4
The estimated parameters for coded variables with significances.

Factor	Estimate	Standard error	Sum of square	*F* ratio	Prob > *F*
Intercept	3.13	0.05	–	–	–
x_1	0.76	0.06	6.92	180.56	<.0001**
x_2	0.33	0.06	1.29	33.78	<.0001**
x_3	0.14	0.06	0.25	6.44	0.0192*
$x_1 \cdot x_2$	0.24	0.10	0.23	5.89	0.0243*
x_3^2	−0.19	0.08	0.24	6.15	0.0217*

x_1, x_2, and x_3 represent the coded variables of lactic acid, chitosan, and rotational rate, respectively. Asterisks indicate statistical significance by *F* test (**$p < 0.0001$; *$p < 0.05$).

where Y ($\log_{10}$ CFU/g) is the log reduction of bacteria, X_1 (%, v/v) is the concentration of lactic acid, X_2 (%, w/v) is the concentration of chitosan, and X_3 (rpm) is the rotational rate.

3.3. Model estimation and validation

The response surface plot in Fig. 1a describes the inactivation effect of lactic acid and chitosan at the fixed rotational rate of 110 rpm (coded level of 0). The population of *V. parahaemolyticus* in shrimps significantly reduced ($p < 0.0001$) as the concentration of lactic acid and chitosan increased, and the bacteria were more sensitive to the concentration of lactic acid than that of chitosan (Fig. 1a). Besides, analysis of variance shows that the interaction between lactic acid and chitosan can play an important role on bacterial inactivation ($p < 0.05$) (Table 4). *V. parahaemolyticus* was only slightly sensitive to the change of rotational rates, as compared with lactic acid and chitosan (Fig. 1b and c). A shoulder shape could be seen at rotational rate above 110 rpm, indicating a level of optimum speed for washing which is in agreement with the significant quadratic coefficient of rotational rate (X_3^2, $p < 0.05$). Treatment time is not a significant factor in the model ($p > 0.05$), suggesting that there are no significant difference of inactivation effect between 10 and 30 min. The estimated optimum treatment conditions were 3% lactic acid combined with 1.6% chitosan for 10 min at 110 rpm, leading to a maximum bacterial reduction of 4.5 $\log_{10}$ CFU/g.

The goodness-of-fit of a predictive model is usually characterized by the root mean square error (RMSE, $\log_{10}$ CFU/g), which was 0.196 in our model, indicating its good statistical performance. However, a statistical validation is insufficient to evaluate accuracy of the model to predict microbial behavior. Therefore, an external validation was carried out. The results of eight independent experiments (not included in the model development) shown in Table 3 were used to calculate the bias factor (B_f) and accuracy factor (A_f) based on Eqs. 3 and 4. The calculated B_f was 1.01, which lies in the acceptable range of 0.9–1.05 proposed by Ross (1996). The A_f value was 1.05, revealing a merely 5% difference between the observations and predictions. These results indicate that the developed model could give a reliable prediction for bacterial survival within the range of variables employed.

3.4. Changes in color and pH of shrimps treated with lactic acid and chitosan

The changes of color (ΔL, Δa, Δb) and ΔpH values of the treated shrimps are summarized in Table 5. The values of lightness (ΔL) and redness (Δa) of the shrimps increased ($p < 0.05$) after most washing treatments. The *b* value of the shrimp color did not change with treatments ($p > 0.05$). In general, the color changes were slight and acceptable based on the visual inspection. There were also no significant differences ($p > 0.05$) in pH values of the shrimps before and after washing treatments.

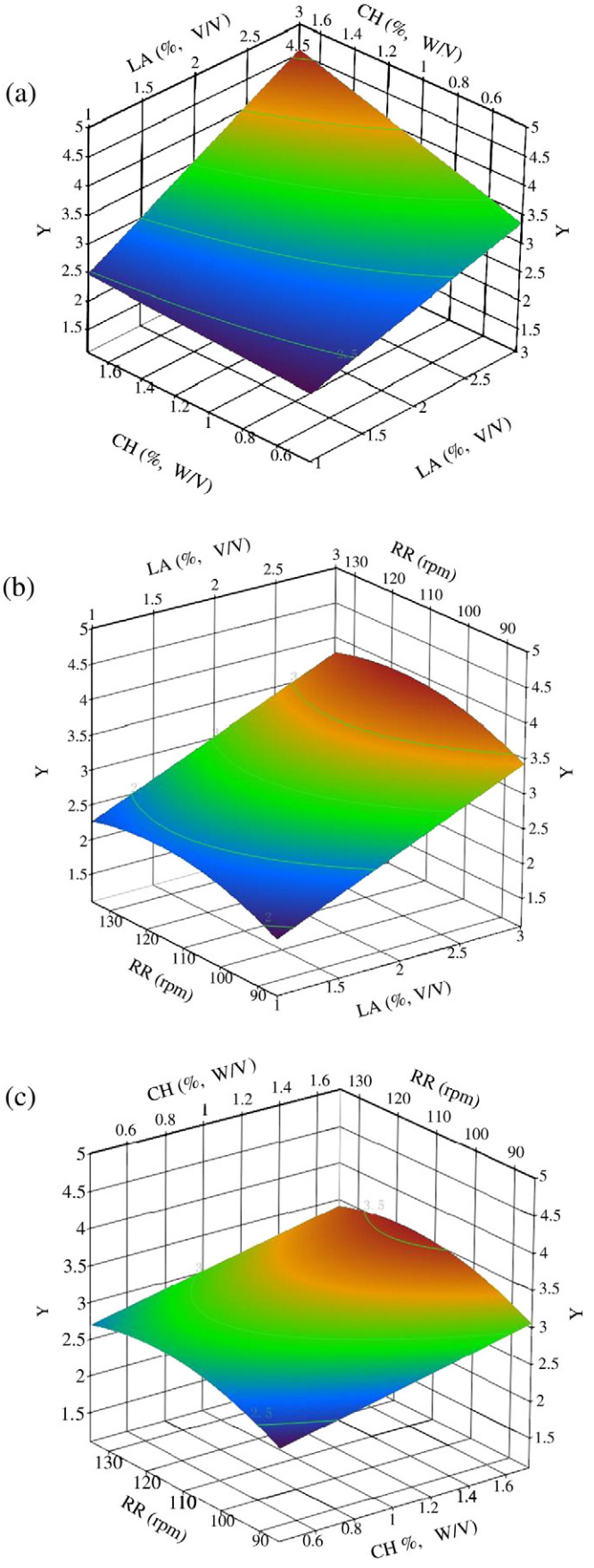

Fig. 1. Response surface plots describing the inactivation effect of lactic acid and chitosan (a); lactic acid and rotational rate (b); and chitosan and rotational rate (c) on *Vibrio parahaemolyticus* in shrimps. *Y* is log reduction of bacteria ($\log_{10}$ CFU/g).

Table 5
Changes in color and pH of the shrimps treated with lactic acid and chitosan washing.

Lactic acid (%, v/v)	Chitosan (%, w/v)	Rotational rate (rpm)	Time (min)	Color			ΔpH
				ΔL	Δa	Δb	
2	1	90	10	0.67 ± 1.42	1.08 ± 1.05	−1.21 ± 1.59	−0.23 ± 0.06
1	1	90	20	2.9 ± 3.35	0.48 ± 0.4	−1.81 ± 0.34	−0.27 ± 0.17
2	0.4	90	20	1.74 ± 1.85	0.49 ± 0.78	−0.89 ± 1.73	−0.08 ± 0.02
2	1.6	90	20	9.36 ± 1.88	0.98 ± 1.17	0.23 ± 1.1	−0.26 ± 0.28
3	1	90	20	3.74 ± 3.82	−1.05 ± 0.55	−3.26 ± 1.65	−0.22 ± 0.01
2	1	90	30	16.39 ± 2.42	2.94 ± 2.38	2.66 ± 2.45	−0.24 ± 0.07
1	1	110	10	5.4 ± 0.65	0.18 ± 1.03	−2.94 ± 3.13	−0.16 ± 0.06
2	0.4	110	10	15.35 ± 1.34	3.3 ± 1.83	−2.94 ± 3.13	−0.1 ± 0.13
2	1.6	110	10	12.01 ± 3.4	−0.49 ± 1.47	0.19 ± 4.4	−0.31 ± 0.25
3	1	110	10	12.59 ± 1.74	1.97 ± 1.12	−2.05 ± 1.13	0.1 ± 0.04
1	0.4	110	20	19.7 ± 0.6	2.11 ± 1.09	0.08 ± 2.31	−0.1 ± 0.08
1	1.6	110	20	4.12 ± 4.68	0.15 ± 0.23	0.96 ± 1.2	−0.08 ± 0.06
2	1	110	20	16.82 ± 2.53	2.23 ± 0.59	0.27 ± 1.21	−0.13 ± 0.04
3	0.4	110	20	18.66 ± 2.72	4.12 ± 0.86	0.61 ± 1.95	−0.16 ± 0.15
3	1.6	110	20	15.89 ± 2.73	4.36 ± 0.65	2.95 ± 2.55	−0.29 ± 0.1
1	1	110	30	12.05 ± 0.73	−0.47 ± 0.75	2.41 ± 1.86	−0.16 ± 0.07
2	0.4	110	30	22.2 ± 0.42	3.92 ± 3.39	−1.9 ± 0.95	−0.08 ± 0.05
2	1.6	110	30	17.9 ± 1.08	2.47 ± 0.76	4.63 ± 2.92	0.09 ± 0.08
3	1	110	30	21.76 ± 2.24	1.88 ± 0.69	2.14 ± 0.89	−0.2 ± 0.06
2	1	130	10	12.32 ± 3.46	0.51 ± 0.87	−1.82 ± 0.96	−0.25 ± 0.09
1	1	130	20	10.63 ± 2.51	−1.05 ± 0.07	−3.49 ± 1.08	0.03 ± 0.06
2	0.4	130	20	21.39 ± 2.55	2.61 ± 1.24	3.48 ± 1.69	−0.16 ± 0.17
2	1.6	130	20	12.08 ± 4.07	−0.34 ± 0.43	−1.6 ± 2.37	−0.2 ± 0.14
3	1	130	20	20.65 ± 1.98	2.47 ± 1.3	1.75 ± 0.9	−0.22 ± 0.14
2	1	130	30	21.54 ± 1.98	2.41 ± 1.09	1.89 ± 1.43	−0.08 ± 0.2

Values represent means ± standard deviations (n = 3).

4. Discussion

In previous studies, lactic acid has been considered as a potential decontamination agent for eliminating pathogenic bacteria in food by acting to permeabilize bacterial outer membrane and to sensitize the bacteria to detergents or lysozyme (Alakomi et al., 2000; Ricke, 2003; Jimenez-Villarreal et al., 2003; Mani-López et al., 2012). Nevertheless, treatment with high concentration of lactic acid for a long time should be avoided in seafood processing due to unacceptable sensory quality (Prakash et al., 2000). Marshall and Kim (1995) suggested that concentrations of lactic acid greater than 2% or exposure time longer than 30 s were not recommended for catfish fillets due to significant color damage. Shirazinejad et al. (2010) reported that dipping in 3% lactic acid for 10 min could reduce *V. parahaemolyticus* in shrimps by 2.5 $\log_{10}$ CFU/g, while a significant change of sensory characteristics ($p < 0.05$) was seen when extending the dipping time to 30 min.

In our study, color changes of treated shrimps were shown as increases of lightness (ΔL) and redness (Δa), leading to a slightly brighter and redder appearance more appealing to visual inspection. The reason suggested for color changes is the denaturation of astaxanthin, the major carotenoid pigment of shrimp (Kaur et al., 2013; Niamnuy et al., 2007). Combined use of lactic acid and higher concentration of chitosan prevented intense discoloration of shrimps by avoiding low pH. The values of ΔL and Δa were significantly lower (indicating better sensory quality) for the combined use of 1% lactic acid and 1.6% chitosan than those of the combination of 1% lactic acid and 0.4% chitosan (pH 4.59 vs pH 2.93).

Bacterial destruction by chitosan is characterized by leakage of proteinaceous and other organelles from bacterial cells, most possibly induced by the reaction of positively charged chitosan molecules and negatively charged cell membranes (Young et al., 1982; Papineau et al., 1991; Ricke, 2003). However, its inactivation efficacy in seafood seems to be low. Chaiyakosa et al. (2007) found that treatment with 1,000 ppm of chitosan for 120 min caused only less than 0.5 $\log_{10}$ CFU/ml reduction of *V. parahaemolyticus* in naturally contaminated shrimps. Terzi and Gucukoglu (2010) decontaminated *V. parahaemolyticus* in mussel meat with reduction by 1.3 to 2.0 $\log_{10}$ CFU/g using 0.05 to 0.5% chitosan for 5 min. Kong et al. (2010) suggested that the primary antibacterial mechanism of chitosan could be due to electrostatic interactions between its polycationic structure and the predominantly anionic components on the bacterial surface, when the environmental pH is below pK_a of chitosan (the amino group in chitosan has a pK_a value of ~6.5). In our research, the pH of lactic acid solutions containing different levels of chitosan is between 2.33 and 4.59, a condition that may result in domination of protonated chitosan with high density of positive charges readily available to bind to negatively charged surfaces, leading to an interactive effect on bacterial inactivation. We found that chitosan alone had a limited effect on *V. parahaemolyticus* inactivation in our preliminary experiment that only 0.6, 0.6 and 0.8 $\log_{10}$ CFU/g reduction were achieved by washing with 1% chitosan for 10, 20 and 40 min at 110 rpm, respectively. Combination of 2% lactic acid with 1% chitosan for 20 min at 110 rpm reduced the bacterial population by 3.0 $\log_{10}$ CFU/g (Table 2), showing the improved inactivation effect of combined treatments compared to either agent alone (2.2 $\log_{10}$ CFU/g reduction for 2% lactic acid, and 0.6 $\log_{10}$ CFU/g decrease for 1% chitosan as shown in our preliminary experiment) in inactivating *V. parahaemolyticus* in shrimps. The developed response surface model also showed a significant interaction between lactic acid and chitosan for enhanced bacterial inactivation.

The analysis of variance reveals that duration of treatment (10, 20 or 30 min) was not a significant factor ($p > 0.05$) for bacterial reduction, suggesting that 10 min or less was enough for decontamination of *V. parahaemolyticus* in shrimps. This is in agreement with an earlier report showing that dipping fresh raw shrimps into 3% lactic acid for 10, 20 and 30 min could achieve *V. parahaemolyticus* population reduction of 2.6, 2.6, and 2.5 $\log_{10}$ CFU/g, respectively, without significant difference ($p > 0.05$) between different treatment times (Shirazinejad et al., 2010). The reason why this shorter duration was better might be due to the fact that both lactic acid and chitosan are bacteriostatic, not bacteriocidal. Bacteriostatic agents, such as chitosan, are more concentration-dependent (Matthew and Levison, 2004; Kumirska et al., 2011). With regard to model validation, we used bacterial counts observed versus predicted for calculation of A_f and B_f which were initially used for comparison of time-based parameters, such as generation time and growth

rate (Ross, 1996). This approach has been used in several recent publications (Bover-Cid et al., 2012; Geysen et al., 2005; Koseki and Yamamoto, 2007; Skandamis and Nychas, 2000) showing that bacterial count was the result of time-based parameters (growth rate, inactivation rate, generation time, etc.) given by the secondary models, which were integrated into the primary model to generate an overall model.

The regulations for the control of *V. parahaemolyticus* in seafood processing vary in different countries. The Codex Committee on Fish and Fishery Products (WHO/FAO, 2007) proposed that the risk reduction could be estimated when the total number of *V. parahaemolyticus* or the number of pathogenic *V. parahaemolyticus* ranges from absence in 25 g to 1,000 CFU or MPN per gram in mollusks. According to the *V. parahaemolyticus* control plan established by the U.S. Food and Drug Administration (FDA), the control measure used in the post-harvest processing should achieve 2 to 3 log reduction of total *V. parahaemolyticus* for oysters from different coastal areas (FDA, 2011a). With the developed model in this study, the estimated bacterial reduction of 4.5 $\log_{10}$ CFU/g could be achieved under optimized treatment conditions (3% lactic acid, 1.6% chitosan, and rotational rate at 110 rpm), which meets the FDA guideline. The simplified response surface model presented in this study has the capability to predict the decontamination effect of lactic acid and chitosan on *V. parahaemolyticus* in shrimps. The combination of lactic acid and chitosan could be potentially used to mitigate the risk of *V. parahaemolyticus* load in shrimps or possibly other seafood products in seafood processing without causing adverse effects on quality attributes.

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