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Acid and Heat Tolerance of Acid-stressed *Listeria monocytogenes* Inoculated in Broth and Shrimp Model

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The acid and heat tolerance of *Listeria monocytogenes* mixed strains were examined after induced to be an acid-stressed cells by culturing in tryptic soy broth supplement with yeast extract pH 5.5 for 1 h. The acid-stressed cells showed better tolerance to acid than that non acid-stressed cells ($p<0.05$) when tested in medium acidified to pH 3.5. For heat tolerance in broth model, both acid- and non acid-stressed cells were centrifuged, suspended in medium at pH 7.2, and heated in shaking water bath at 55-63°C for 0-180 min. D-value of acid- and non acid-stressed cells at 55, 57, 59, 61 and 63°C were 45.25, 17.83, 7.47, 3.80 and 2.40 min and 34.36, 11.96, 7.37, 3.74 and 2.42 min, respectively. In addition, Z-value of acid- and non acid-stressed cells were different at 6.21 and 7.08°C, respectively. From these results, acid-stressed cells showed cross protection effect higher than that non acid-stressed cells. However, heat tolerance of acid-stressed cells inoculated on Pacific white shrimp flesh using sous vide technique (packed in vacuum pouch, and heated at 60, 62.5 and 65°C for 10 min) did not show a significant difference when compared with non acid-stressed cells ($p>0.05$). While blanching inoculated shrimp flesh at 70°C for 2 min, non acid-stressed cells survived more than acid-stressed cells. The results revealed that acid-stressed cells showed acid and heat tolerance better than non acid-stressed cells especially in medium, not in shrimp.

Key words: Acid-stressed cells, *Listeria monocytogenes*, shrimp, acid tolerance, heat tolerance.

Listeria monocytogenes is a food-borne pathogen that is distributed in a wide variety of environment and food products. It has an adaptive response for its survival after an encounter with environmental stresses. One of the significant environmental stresses which *L. monocytogenes* faced during live in soil, foods, and human is an acid stress. When facing with sublethal acidic environmental, bacteria will alter their physicochemical to survive, which was called acid adaptation. Acid adaptation not only enhanced the viability of *L. monocytogenes* after exposing with lethal acidic condition[1-3] but also increased the survival of *L. monocytogenes* after exposing with heat, cold, salt, sanitizer (chlorine, quaternary ammonium compound, hydrogen peroxide, and ethanol), bacteriocin, and UV radiation when compared with non acid-stressed cells [2,4-9]. Shrimp was reported as a vehicle of listeriosis outbreaks[10]. The acid adaptation and survival of *L. monocytogenes* in food products especially in shrimp are rarely studied. The objective of this work was to evaluate the effect of acid and heat treatment on the survival of acid-stressed *L. monocytogenes* that inoculated in broth and shrimp sous vide.

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MATERIALS AND METHODS

Shrimp preparation

Pacific white shrimp (Litopenaeus vannamei) of weight about 50 kg was purchased from a farm collection center. Shrimp was placed in crushed ice with a shrimp/ice ratio of 1:3 (w/w) and transported under ice to the Department of Food Science and Technology, Kasetsart University within 3-4 h after purchased. Then they were washed with tap water, peeled, deveined and head off. After that all shrimp meat were individual pack into low density polyethylene bag and stored at -18°C until used. Prior to the beginning of each experiment, frozen shrimp in a bag were placed through the running tab water until the shrimp temperature reaches to 10°C.

Bacteria strains and culture preparation

The strains of *L. monocytogenes* used in this study included 101, 108, 310, V7 and Scott A. All of these strains were preserved by freezing the cultures at -30°C in vials containing trypticase soy broth (Difco, USA) with 0.6% yeast extract (Difco, USA) (TSBYE) supplemented with 20% (vol/vol) glycerol (Merck, Germany). Working culture were prepared by thawing stock culture at ambient temperature, and taking one loop full into 10 ml of TSBYE and incubating at 35°C for 24 h two times. Then, 1 ml of these cultures were transferred to 100 ml TSBYE and incubated for 18 h as working culture.

Induction acid-stressed cells of *L. monocytogenes*

A five-milliliter aliquot of each working culture strain was combined in a sterilized conical 50-ml tube, mixed, centrifuged (Beckman Coulter model J2-MC centrifuge) at 4,500 x g for 15 min, 28°C (room temperature) and washed twice with 20 ml of 0.1 M sterile phosphate-buffered saline (PBS; 0.2g of KH_2PO_4 (BDH, UK), 1.5 g of $Na_2HPO_4.7H_2O$ (Merck, Germany), 8.0 g of NaCl (Merck, Germany), and 0.2 g of KCl (Merck, Germany) in 1 liter of distilled water and adjusted pH to 7.4 with a pH meter (Orion Research, USA)) in order to remove metabolic end products. Cells suspension was then resuspended in 25 ml of TSBYE and diluted with TSBYE to yield a population of 10^7 CFU/ml. Then, an aliquot (1 ml) was transferred to 100 ml of TSBYE, pH 5.5, adjusted with lactic acid (JT Baker, USA) (acid-stressed cells) or 100 ml of TSBYE, pH 7.2 (non acid-stressed cells), and incubated for 1 h at 35°C. Initial cell populations were enumerated by using spiral plating technique (Autoplate 4000; Spiral Biotech, Bethesda, MD, USA), plating on trypticase soy agar (TSA; Merck, Germany) or modified Oxford agar (MOX; Oxoid, UK) and incubating at 35°C, 48 h.

Acid tolerance

Broth After exposure to acid stress condition of culture broth, both acid-stressed cells and non acid-stressed cells were centrifuged at 4,500 x g and washed two times with PBS. Then cells pallets were suspended in 100 ml TSBYE pH 3.5 (adjusted with hydrochloric acid (Merck, Germany)) and incubated at 35°C to assess the acid tolerance of cells. The aliquots (1 ml) were removed at 0, 0.5, 1, 1.5, 3, and 6 h and diluted with 9 ml of PBS and 0.1 ml of the suitable dilution was surface plated on duplicate plates of TSAYE. The plates were incubated at 35°C for 48 h, after that colonies were counted. Two replicates of the experiments with two samples analyses in each treatment were analyzed.

Heat tolerance

Broth Triplicate flasks with 100 ml of TSBYE (pH 7.2) were heated in thermostatically controlled shaking water baths until the medium reached 55, 57, and 60°C. The level of water in the water baths was 2 centimeters above the level of heated medium in the flasks. Then 1 ml of acid-stressed cells and non acid-stressed cells suspension were inoculated into the TSBYE. Samples were heated at 55, 57, and 60°C for 90, 60, and 20 min, respectively and samples (1 ml) were withdrawn with appropriate interval. Then, the samples were plated on duplicate plates of TSAYE incubated at 35°C for 48 h. and colonies were counted. Two replicates of the experiments with two samples analyses in each treatment were conducted. D and z-value were calculated.

Shrimp Shrimp was surface inoculated with acid-stressed cells or non acid-stressed cells of *L. monocytogenes* by dipping with the culture at a ratio of 2:1, and then stirred with sterilized tongs for 5 min in a sterile beaker. Shrimp was placed on a sterilized wire rack in order to drain excess inoculums for 15 min. Inoculated shrimps (both acid- and non acid-stressed cells) were divided into 2 groups in order to study the effect of vacuum packing (sous vide cooking) and non vacuum packing (conventional cooking). First

group, vacuum packing, the inoculated shrimp were placed in a laminated pouch (Royal Can Industries, Co.Ltd, Thailand), and packed under vacuum by using a vacuum packaging machines (Henkovac, The Natherlands). The sample pouches were placed in the basket and moved into a water cascading retort (Hisaka work, LTD, Japan) with static mode (no basket rotation). Samples were heated at an internal temperature 60, 62.5, and 65°C for 10 min and cooled in retort and ice-water bath for10 and 10 min, respectively in order to reach an internal temperature 3°C. For temperature control, a T-type thermocouple was fixed at the slowest heating point using a stuffing clamp and connects with a data logger (Graphtec Corporation, China) for collecting time-temperature of two samples and retort chamber. Second group, non vacuum packing, the inoculated shrimp were directly treated with 500 ml of water heated at 70°C for 2 min in a ratio of shrimp and water 1:5 and shrimp were cooled in ice-water bath. After that shrimp samples were placed in stomacher bag and stomached with 9 volume of 0.1 M PBS at a medium speed for 2 min (Seward Ltd, UK). The stomached shrimp samples were 10-fold serially diluted using PBS and taken 0.1 ml of each sample plating on duplicate plate of TSAYE and MOX and incubated at 35°C for 24 and 48 h, respectively in order to count total bacteria and Listeria in shrimp sample.

Texture analysis for shrimp meat

Texture was evaluated on a universal testing machine (Stable Micro Systems, Surrey, England). Shear force was evaluated using the Warner–Bratzer shearing device attached to the load cell (50-kg capacity). To mimic human bite, the shrimp abdomen was cut transversally between the second and third somite; the cross-head speed was 10 cm/min. The first major force peak was recorded as the maximal shear force required to shear the muscle sample.

Colour analysis for shrimp meat

The colour of whole uninoculated shrimps meats (middle portion) was determined in the L*, a*, and b* mode of CIE (angle 10°, illuminant D65) using Hunter Lab (ColorFlex, Hunter Associates Laboratory, VA, USA). L*, a*, and b* indicate lightness, redness D greenness and yellowness D blueness, respectively. The measurement was conducted in six replications.

RESULTS AND DISCUSSION

Acid tolerance

In broth model, after exposure acid-stressed cells of mixed strains of *L. monocytogenes* to mild acid (pH 5.5) TSBYE at 35°C for 1 h, acid-stressed cells increased acid resistance (Table 1). Mild acid stress enhanced the survival of acid-stressed cells after exposing with lethal acid (pH 3.5) TSBYE better than that non acid-stressed cells (control). However, increasing time of exposure to acid reduced the survival of both cells and eliminated both cells after 3 h. Cataldo (11) reported that *L. monocytogenes* LM2 following resuspended in BHI pH 5.1 for 1h at 37°C showed resistance to lethal acid (pH 3.5) more than non acid-stressed cells. Furthermore, Koutsoumanis *et al*. (12) also stated that *L. monocytogenes* mixed strains (Na-4, 163, N-7143, N-71Table 1 44 and N-7159) exposure to sublethal TSBYE pH 5.0 and 5.5 for 90 min at 30°C are more resistant to pH 3.5 than that TSBYE pH 6.0, 4.5, 7.0 and 4.0, respectively. Moreover, the death rate of acid-stressed cells of *L. monocytogenes* at pH 5.5 is the least (1). In addition, acid-adapted Vibrio vulnificus also increased acid tolerance with increasing adaptation time[13].

Heat tolerance

In broth mode, D- and Z-value of mixed strain of acid-stressed cells and non acid-stress cells of *L. monocytogenes* that expose and non expose to mild acid stress, respectively were shown in table 2. D-value at 55, 57, 59, 61 and 63°C of acid-stressed cells were 45.25, 17.83, 7.47, 3.80 and 2.40 min, while non acid-stressed cells were 34.36, 11.96, 7.37, 3.74 and 2.42 min, respectively. Z-value of acid- and non acid-stressed cells were different as 6.21 and 7.08, respectively. From the result, it was noted that acid-stressed cells of *L. monocytogenes* can adapt and produce the cross protective against thermal tolerance. Agreement with Skandamis *et al.*,[6], they also found that acid-stressed cells of mixed strain (Scott A, NA-3, NA-19, 101M, 103M, 558, PVM1, PVM2, PVM4 and PVM4) of *L. monocytogenes* which exposed with mild acidic TSBYE, pH 5.0 for 1 and 1.5 h at 30°C resisted heating more than that non acid-stressed cells. Leenanon and Drake[14] reported that heat resistance of *Escherichia coli* O157:H7 (ATCC

43895) and nonpathogenic *E. coli* (ATCC 25922) were enhanced after exposure to mild acid condition. In the other hand, antibiotic stress did not increase acid and heat Table 2 olerance of *E. coli* O157:H7 (15).

In shrimp non vacuum packing (conventional cooking) model, blanching inoculated shrimp at 70°C for 2 min resulted in acid-stressed cells and non acid-stressed cells of *L. monocytogenes* reduced 99.99 and 99.75%, respectively. While total bacteria count were reduced 99.09 and 99.41% respectively (Figure 1). From these results, it was found that non acid-stressed cells of both *L. monocytogenes* count and total bacteria count resist to heat more than that acid-stressed cells. Furthermore, after heating amount of *L. monocytogenes* reduce more than that total bacteria count. According to Wu and Fung[16] reported that heating cells suspension of *L. monocytogenes* at 55°C for 10 min caused injury

Table 1. Survival of acid-stressed cells and non acid-stressed cells of *Listeria monocytogenes* after expose to acid (pH3.5)

Exposure time (h)	*Listeria monocytogenes* count (Log CFU/ml)[a]	
	Acid-stressed cells	Non acid-stressed cells
0	4.72 ± 0.15^{Aa}	4.38 ± 0.56^{Aa}
0.5	4.05 ± 0.10^{Ab}	3.42 ± 0.15^{Ba}
1.0	3.04 ± 0.04^{Ac}	2.13 ± 0.19^{Bb}
1.5	2.00 ± 0.19^{Ad}	0.60 ± 0.51^{Bc}
3.0	ND^{Ae}	ND^{Ac}
5.0	ND^{Ae}	ND^{Ac}

[A-B] with in each row of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
[a-e] with in each column of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
[a] are means ± standard deviations from two trials each having triplicate samples.
[b] NB: Non detectable

Table 2. D- and Z-value of acid-stressed cells and non acid-stressed cells of *Listeria monocytogenes* after exposure to different temperatures

Temperature (°C)	Acid-stressed cells D-value (min)	Non acid-stressed cells D-value (min)
55	48.40 ± 4.45^{Aa}	36.49 ± 3.01^{Ba}
57	17.21 ± 0.88^{Ab}	11.86 ± 0.15^{Bb}
59	7.48 ± 0.02^{Ac}	6.65 ± 1.02^{Ac}
61	3.80 ± 0.48^{Ad}	3.84 ± 0.24^{Ad}
63	2.54 ± 0.44^{Ad}	2.42 ± 0.20^{Ad}
Z-value (Temperature; °C)	Z-value (°C) 6.17 ± 0.06^{B}	Z-value (°C) 7.08 ± 0.08^{A}

[A-B] with in each row of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
[a-d] with in each column of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
Data are means ± standard deviations from two trials each having triplicate samples.

of cells which can not growth well in selective media (MOX agar) when compare with non selective media (TSAYE).

In shrimp vacuum packing (sous vide cooking) model, increasing the processing temperature resulted in reduction of acid and non acid-stressed cells of *L. monocytogenes* and total bacteria count (Table 3). Acid-stressed cells of *L. monocytogenes* was reduced 2.91, 4.43, and 4.97 log CFU/g while non acid-stressed cells was reduced 3.05, 4.28, and 4.87 log CFU/g when heating at 60, 62.5, and 65°C, respectively. According to The National Advisory Committee on Microbiological Criteria for Foods (NACMCF), they recommended guidelines for evaluating the ability of thermal processes to inactivate *L. monocytogenes* in extended shelf life refrigerated foods should provide a heat treatment which is sufficient to achieve a 4 decimal log reduction (4D) of *L. monocytogenes*[17]. From the results, it was found that all samples heating at 62.5 and 65°C for 10 min reached this NACMF's recommend and it can imply that heated 62.5 and 65°C for 10 min, shrimp product will safe for consumed.

The colour (L*, a*, and b*-values) of white shrimp meat heating at different temperatures was shown in Table 4. From the results, all of the colour, lightness (L*), redness (a*), and yellowness (b*), of shrimp heated at different temperature were significantly higher than that fresh shrimp. However, the color values of shrimp heating at all temperature were not significant difference ($p > 0.05$). According to Benjakul *et al*. (18) reported that heating shrimp by streaming up to 3 min (core temperature 70-75°C) increased L*, a*, and b* value when compare with fresh shrimp. However, L*, a*, and b* value of shrimp heated for 2 and 3 min did not significantly different. The results noted that heating resulted in changing color of shrimp since carotenoproteins were denatured and released carotenoids which have orange to red which increased both a* and b* value.

Shear force of shrimp meat from different heating temperatures was shown in table 5. The

Table 3. The survival of *Listeria monocytogenes* and total bacteria after processing at different temperature

Heating temperature	Processing time	*L. monocytogenes* (Log CFU/g: MOX)		Total bacterial count (Log CFU/g: TSAYE)	
(°C)	(min)	Acid-stressed cells	Non acid-stressed cells	Acid-stressed cells	Non acid-stressed cells
0	0	5.97±0.10[a]	5.87±0.06[a]	5.94±0.08[a]	5.98±0.05[a]
60	10	3.06±0.80[b]	2.82±0.27[b]	3.16±0.68[b]	2.74±0.68b
62.5	10	1.54±0.43[c]	1.59±0.00[c]	2.30±0.28[bc]	2.17±0.11[bc]
65	10	<1[d]	<1[c]	1.40±0.57[c]	1.35±0.00[c]

[a-d] with in each column of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
Data are means ± standard deviations from two trials each having triplicate samples.

Table 4. Color change of shrimp during processed at different temperature for 10 min

Heating temperature	color			Shear force
(°C) for 10 min	L*	a*	b*	(N)
Fresh shrimp	56.16±2.35[a]	-0.65±0.57[a]	3.27±0.84[a]	19.44±2.47[ab]
60	74.86±2.41[b]	15.83±2.07[b]	17.22±1.69[b]	20.53±1.96[a]
62.5	74.69±1.36[b]	17.55±1.91[b]	19.21±1.74[b]	17.79±1.37[b]
65	76.82±1.38[b]	16.37±1.84[b]	19.07±1.49[b]	19.84±1.85[ab]

[a-d] with in each column of the test samples, means followed by the same letter are not significantly different from each other at P = 0.05.
Data are means ± standard deviations from two trials each having triplicate samples

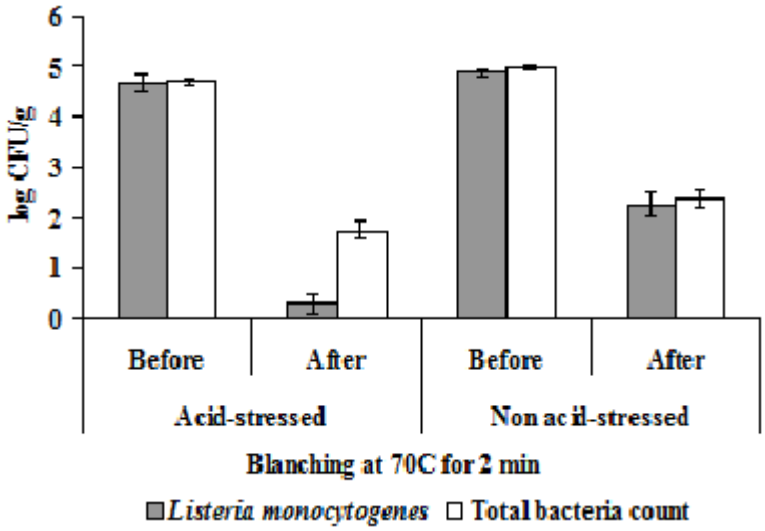

Fig. 1. Changing amount of bacteria of shrimp that inoculated with acid- or non acid-stressed cells of *Listeria monocytogenes* on before and after blanching at 70°C for 2 min. ■, *Listeria monocytogenes*; □, Total bacteria count

shear force of shrimp meat did not show clear tendencies similarly report of Schubring[19] who study the effect of heating temperature (30-80°C) on tenderness of brown shrimp, northern shrimp, and deepwater pink shrimp meat. He found that the tenderness of almost shrimps was not increased when increasing temperature up to 50°C. After increasing temperature over 50°C, brown and deepwater pink shrimp meat become softer and harder again. Many researchers found that the shear force of cooked shrimp affected by heating temperature (19,20) and heating time[18,21]. In addition, increasing heating temperature increased cooking loss and decreased cooking yield[22].

CONCLUSION

The acid-stressed cells showed the better resistance to the challenge conditions as acid, and heat over the non acid-stressed cells did. According to Lou and Yousef [2] and Koutsuoumanis and Sofos[1] found that sublethal acid stress induces tolerance to lethal acid stress. Cross-tolerance to thermal stress was also reported by Faber and Pagotto[23] and Skandamis *et al.*,[6]. However, heat resistance of acid-stressed cells of *L. monocytogenes* did not show different from that non acid-stressed cells when inoculated on shrimp, especially heating at 65°C in vacuum packing (sous vide cooking), both of acid- and non acid-stressed could eliminate to undetected. In addition, the colour and texture of sous vide cooking at 65°C did not different from that cooking at 60 or 62.5°C. The results implied that sous vide cooking at 65°C could make shrimp safe from contamination of both acid-stressed and non acid-stressed cells of *L. monocytogenes*. These results may be a guide for shrimp processing industries.

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