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The Effects of Heat Shock on the D-Values of *Listeria monocytogenes* on Selected Seafood Matrices

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Abstract

With more ready-to-eat foods and increased shelf-lives, prevention of *Listeria monocytogenes* contamination has become a necessity for the food industry. This study examined the effects of sublethal heat treatment on the decimal reduction time (D-values) of three *L. monocytogenes* serotypes (1/2a, 1/2b, 4c), and non-pathogenic *L. innocua*. The D_{70} (D-value at 70˚C) values of heat-shocked (HS) and non-heat-shocked (NHS) *Listeria* grown in tryptic soy broth (TSB) were determined. The D_{70} values of HS *L. monocytogenes* serotype 1/2a and *L. innocua* were significantly higher compared to NHS cultures, although by 48 h, the values returned to NHS levels. When HS and NHS 1/2a and 1/2b were inoculated on crab meat and cooked shrimp, the D_{70} values of HS cultures were at least 2-fold higher, compared to when they were grown in TSB. This increase in heat resistance for the HS cultures may be attributed to the protective effect of the seafood matrix itself.

Keywords

***Listeria monocytogenes*, Ready-to-Eat Seafood, Heat Shock, D-Value**

1. Introduction

In the past several decades, listeriosis, caused by the intracellular pathogen *Listeria monocytogenes*, has emerged as a major foodborne disease worldwide [1]-[3]. Of the eight current species in the genus *Listeria*, *L. monocytogenes* is the only one considered to be a foodborne pathogen in both humans and animals [4]. *Listeria*

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monocytogenes is divided into four distinct lineages each with unique genetic, phenotypic, and epidemiological characteristics. Lineages I and II are both associated with human disease, although most human listeriosis isolates are from lineage I. Lineage II strains are more frequently associated with sporadic listeriosis in humans, but are also associated with animal listeriosis. Lineage III and IV isolates are rarely found in foods or food-related environments, and have not been linked to human listeriosis [5]. Serovars 1/2a (lineage II), 1/2b and 4b (lineage I) account for 95% of human isolates [6].

Listeria has a potential to grow during cold storage due to its ability to proliferate at low temperatures (0 to 45˚C) and reduced oxygen levels, and is a problematic microorganism in the ready-to-eat (RTE) seafood industry. Several listeriosis outbreaks have been associated with seafood products [7]-[9], and *Listeria* spp. has been detected in several seafoods including fresh shrimp, crab meat, and smoked and fermented fish [3] [10] [11]. For RTE crustaceans, specifically cooked shrimp and canned crab meat, the risks are primarily linked to post-process contamination [12]. Further, studies have shown that some cold-smoking processes do not completely kill *L. monocytogenes* [7]. The ability of smoked seafood to support the growth of *Listeria* during extended refrigeration temperatures [9] and the prior instances of outbreaks due to improper cooking times and temperatures during processing [7] accentuate the risk of *Listeria* contamination in RTE seafood.

Previous studies have shown that certain strains of *L. monocytogenes* exhibit a higher decimal reduction time (D-value) than others [13]. Differences in D-value, which is the time required to reduce a bacterial population by 90% (1.0 log unit) at a given temperature, can be attributed to various factors that contribute to pathogenicity [14]. The time of exposure to heat shock, the heating matrix, the condition of the *L. monocytogenes* culture, as well as the method used to recover the cells all have an effect on bacterial thermotolerance [15]. A previous study found that heat shocking *L. monocytogenes* at 42˚C for 60 min, followed by 60˚C for various lengths of time resulted in approximately twice the D_{60}-values compared to the non-heat shocked strain [16].

Several studies have investigated the effect of heat shock proteins on the D-values of certain strains of *L. monocytogenes* in certain types of food including dairy products, and ham [13] [16]. However, limited information exists regarding the relationship among the D-values of heat shocked and non-heat shocked *Listeria* spp. on RTE seafood. This study examined three serotypes of *Listeria monocytogenes*, 1/2a, 1/2b, and 4c, and one strain of *Listeria innocua*, a non-pathogenic species, to determine how the heat shocking affected D-values in both growth media and inoculated RTE seafood.

2. Material and Methods

2.1. Bacterial Cultures

Listeria monocytogenes cultures DUP-1039C (1/2a), DUP 1042B (1/2b) and 758453 (4c), originally isolated from smoked fish, a human isolate, and an animal isolate, respectively, were obtained from Dr. M. Weidman (Cornell University). *Listeria innocua* (ATCC 33090) was obtained from Dr. A. Wright (University of Florida). All four *Listeria* cultures were adapted to 350 $mg \cdot l^{-1}$ nalidixic acid (NX), to eliminate contamination from background microflora during bacterial recovery and enumeration.

2.2. Seafood Products

Seafood matrices used in this study, crab meat (*Callinectes sapidus*), raw shrimp (*Penaeus setiferus*) and smoked salmon (*Oncorhuncus goruscha*) were commercially obtained from Northwest Seafood, Gainesville, FL. Crab meat and salmon were processed prior to purchase, whereas the raw shrimp was boiled for five min in tap water and refrigerated before the study. All seafood samples were cut into 10 g pieces.

2.3. Heat Shocking of *Listeria* Cultures and Determination of D_{70}-Values

Before heat shocking, two sets of *Listeria* cultures were grown in 10 ml of tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD)/NX (50 $mg \cdot l^{-1}$) at 37˚C overnight. Ten µl of culture was transferred into fresh TSB for four consecutive days, and incubated at 37˚C overnight. On day five, the bacterial cultures were centrifuged (4000 g for 20 min), washed twice and resuspended in fresh Universal Pre-Enrichment Broth (UPB) (Difco, Becton Dickinson, Sparks, MD), to yield a concentration of 1.0×10^7 cfu/ml. The cultures were initially exposed to a temperature of 50˚C (simulating seafood temperature abuse conditions before processing), to evaluate the effect of sublethal heat treatment on the ability of *Listeria* strains to withstand lethal heat treatment at 70˚C. A

subsequent thermal inactivation temperature of 70˚C (D_{70}-value) was chosen based on typical temperatures recommended for many smoked seafood products [17]. The cultures were initially subjected to a temperature of 50˚C by immersing the tubes in a waterbath for 60 min (Precision Series 280, Winchester, VA), and then given 15 - 30 min to recuperate [15]. One set of cultures were then incubated at room temperature (non-heat shocked), while the other (heat shocked) underwent the heat shocking protocol as described previously [15]. Briefly, the sublethally heated samples were placed into a waterbath (Precision Series 280, Winchester, VA), to heat up to 70˚C for a total time of 5 and 5.5 min (with a 30 s warm up time). Heat shocked cultures, along with the corresponding non-heat shocked cultures, were removed every 30 s for 5 min, and immediately placed on ice to stop any further heat inactivation. For enumeration, samples were serially diluted, pour plated on TSA, and incubated for 18 to 24 h at 37˚C. D_{70}-value reversion was also calculated to evaluate the reversion of heat shocked cultures back to the non-heat shocked state. For this purpose, all HS and NHS cultures were inoculated into fresh TSB and incubated at 37˚C for 24 and 48 h, and D_{70} values were determined. An initial time point of 24 h for reversion studies represents the typical refrigerated storage times for food in households [18]. The experiment was repeated three times. The $\log_{10}$ values for cell counts were plotted against the time of exposure, and linear regression analysis was performed to calculate the D_{70}-values. The data was statistically analyzed for significant differences by analysis of variance (ANOVA) and least squares difference test ($p \leq 0.05$) using SAS (SAS Institute, Cary, NC, USA).

2.4. Inoculation of Seafood Products and Determination of *Listeria* D_{70}-Values

Only the pathogenic strains, *L. monocytogenes* 1/2a and 1/2b were included in studies for the determination of D_{70}-values in seafood matrices. One hundred µL (1.0×10^6 cfu/ml) of heat shocked or non-heat shocked cultures were pipetted onto each 10 g seafood sample (cooked shrimp, crab meat, and smoked salmon). Samples incubated for 60 min at room temperature to achieve bacterial attachment. Seafood samples were placed into a Whirl-Pak® bag (NASCO, Fort Atkinson, WI), and placed into the Precision Series 280 waterbath and heated to an internal temperature of 70˚C for 5 min (with a warm up time of 30 s). The samples were removed every 30 s for 5 min, and immediately placed on ice to stop any further heat inactivation. For *Listeria* enumeration, 90 ml of PBS was added to each sample. Samples were serially diluted in PBS and pour-plated in TSA/NX. Plates were incubated at 37˚C for 18 to 24 h before enumeration, and D_{70}-values calculated as described previously.

3. Results and Discussion

3.1. D_{70}-Value Determination for Heat Shocked Cultures

Heat shocking significantly increased the D_{70}-values of *L. monocytogenes* 1/2a and *L. innocua* on day 0 (**Table 1**), but not that of *L. monocytogenes* 1/2b and 4c. By 48 h, the D_{70}-values of all HS *Listeria* cultures reverted back to that of NHS cultures (**Table 1**).

Results from this study revealed that D_{70}-values of *L. monocytogenes* 1/2a was much higher when subjected to heat shock conditions, compared to the D_{70}-values of the non-heat shocked cultures. The D-value reversion findings suggest that heat shocked *L. monocytogenes* cultures have the ability to recover from non-lethal heat treatments after 24 h once recultured into fresh enrichment broth. The increase in D_{70}-values brought on by heat shocking fell significantly after 24 h. This study showed that although sub-lethal thermal injury can induce heat

Table 1. D_{70}-values (min) of *Listeria* cultures grown in tryptic soy broth, before and after heat shocking, on day 0, 24 h and 48 h.

***Listeria* culture**	Time and Treatment			
	NHS (Day 0)	**HS (Day 0)**	**HS (24 h)**	**HS (48 h)**
1/2a	1.17 ± 0.0 a	2.32 ± 0.1 c	1.90 ± 0.6 bc	1.46 ± 0.1 ab
1/2b	1.31 ± 0.2 a	1.47 ± 0.2 a	0.99 ± 0.1 a	1.11 ± 0.1 a
4c	1.29 ± 0.1 a	1.56 ± 0.1 a	1.11 ± 0.0 a	1.3 ± 0.0 a
innocua	0.82 ± 1.1 a	2.5 ± 0.3 b	0.85 ± 0.0 a	0.81 ± 0.0 a

*Values with different letters (a-b) in the same row differ significantly ($p < 0.05$).

shock protein production and a subsequently increase D_{70}-values, these effects are lost after 24 - 48 h. Previous research has also shown that the protective responses induced by heat shocking are reversible [19] [20].

3.2. D_{70}-Values and Seafood

A preliminary recovery study showed no statistical difference between inoculated and recovered levels of *Listeria* from any of the seafood matrices (**Table 2**). Additionally, a background microflora study showed that no organisms native to cooked shrimp, crab meat or smoked salmon had the ability to grow on the TSA/NX media used in this study (data not shown). The D_{70}-values were calculated by using standard regression analysis coupled with a standard ANOVA analysis to determine statistical differences between the cultures. There was a significant increase in heat resistance (≥2-fold) for the HS cultures (1/2a and 1/2b) inoculated on cooked shrimp and crab meat. However, the D_{70}-values of NHS and HS *L. monocytogenes* 1/2a and 1/2b inoculated on smoked salmon were not significantly different (**Table 3**). The D_{70}-value of HS *L. monocytogenes* 1/2a in TSB was 2.3 min, while the average D_{70}-value of the same organism inoculated on the experimental seafood matrices was 3.2 min. For the HS *L. monocytogenes* 1/2b, the D_{70}-value in TSB was 1.5 min, compared to 3.4 min for the average D_{70}-values of HS *L. monocytogenes* 1/2b on the tested seafood matrices.

When shrimp and crab meat were inoculated with heat shocked (HS) and non-heat shocked (NHS) pathogenic *Listeria* cultures, a two-fold or more increase in the D_{70}-values were observed. This increase in heat resistance when compared to enrichment broth, could be attributed to the protective effect of the seafood matrix itself. The protective effect of the food matrix as observed in this study also concurs with another thermal inactivation study [13], in which the D_{60}-values of *L. monocytogenes* inoculated in cream was two-fold higher than the D_{60}-values in TSB. Further, the D_{60}-values were also four times higher in the cultures inoculated on the seafood matrices than in TSB [21]. When comparing D_{70}-values for each of the seafood matrices in this study, crab meat showed the most protective effect, showing a ≥2.5-fold increase in D_{70}-values compared to the values in TSB. The D_{70}-values for cooked shrimp increased by ≥2-fold, but that of smoked salmon increased only by ≥1.2-fold. A previous study conducted by [21] also noted an increase in D-values associated with *Listeria* inoculated on seafood, and concluded that fat content had a significant role in the increased survivability. The results from this study however, do not suggest a direct correlation between the fat content and increased thermal resistance for

Table 2. Log reduction during the recovery of *Listeria* strains from inoculated seafood matrices.

Seafood Matrix	*Listeria* Culture			
	1/2a	1/2b	4c	*innocua*
Crab Meat	0.01	0.09	0.17	0.08
Smoked Salmon	0.01	0.01	0.20	0.19
Cooked Shrimp	0.01	0.02	0.09	0.20

Table 3. D_{70}-values (min) of cooked shrimp, crab meat, and smoked salmon inoculated with NHS and HS *L. monocytogenes*.[*]

Seafood Matrix	*Listeria* Culture	Treatment	
		NHS	HS
Cooked Shrimp	1/2a	1.45 ± 0.18 a	2.79 ± 0.15 b
	1/2b	1.41 ± 0.13 a	2.92 ± 0.48 b
Crab Meat	1/2a	1.82 ± 0.15 a	4.62 ± 0.63 b
	1/2b	1.78 ± 0.15 a	5.05 ± 0.62 b
Smoked Salmon	1/2a	1.68 ± 0.09 a	2.22 ± 0.28 a
	1/2b	1.92 ± 0.16 a	2.22 ± 0.28 a

[*]Values with different letters (a-b) in the same row differ significantly ($p < 0.05$).

the matrices examined. Smoked salmon, which had the highest fat content in this study (8 g of fat per serving), yielded the smallest increase in D_{70}-value. This observation is in accordance with a previous study on the heat resistance of *L. monocytogenes* in dairy products, which also concluded that that there was no correlation between fat content and heat resistance [13]. Besides fat content, other factors including water activity and osmotic stress could also have a protective effect yielding an increased thermal resistance [22]-[24]. A previous study by Farber [25] showed that when shrimp, crab and smoked salmon were stored at room temperatures for 6 h (simulating temperature abuse conditions), the growth of *L. monocytogenes* was the lowest on smoked salmon, compared to cooked shrimp and crab. According to the study, the reduced growth of *L. monocytogenes* strain HPB 323 on smoked salmon may potentially be due the sensitivity of the pathogen to sodium chloride or other antimicrobial compounds present in smoke. The results from the current study also indicate that out of the three seafood matrices compared, smoked salmon had the least protective effect on *L. monocytogenes*. Antimicrobial effect of smoke on *L. monocytogenes* has also been reported by previously [26].

4. Conclusion

The results from this study indicate that D_{70}-values of HS *L. monocytogenes* 1/2a and *L. innocua* strains are significantly higher when compared to NHS cultures. However, after 24 - 48 h, a minimum refrigeration period for food before being reheated and consumed (NSF International 2015), the D_{70}-values noted in this study reverted to levels equivalent to those seen prior to heat shocking. Thus, the cross-protection attributed to these injured microorganisms may not be a factor in the virulence of *L. monocytogenes* in food stored long. The higher D_{70}-values of *L. monocytogenes* on contaminated cooked shrimp and crab meat suggest a greater protection for the pathogen from the detrimental effects of heat. Thus, contaminated RTE seafood may need to be heated for a longer time to inactivate *L. monocytogenes*.

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