1 **Effect of Temperature and Salt on Thermal Inactivation of *Listeria***
2 ***monocytogenes* in Salmon Roe[†]**

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7 Running Head: Thermal inactivation of *L. monocytogenes* in salmon roe

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Abstract

Listeria monocytogenes is a potentially fatal foodborne pathogen that can be found in ready-to-eat seafood products, such as fresh salmon roe. Once contaminated, salmon roe must be decontaminated prior to human consumption. This study was conducted to determine the thermal inactivation kinetics of *L. monocytogenes* in raw salmon roe as affected by bacterial strain, temperature, and salt concentration. Three different strains of *L. monocytogenes*, including serotype 4b (F2365), 1/2b (F4260), and 1/2a (V7), were individually inoculated to salmon roe supplemented with salt (0-4.5%), and heated under different temperatures (57.5-65.0°C) to evaluate the survival of the bacterium during heating and determine the D-values. Results showed that the thermal resistance (log D) of *L. monocytogenes* was significantly affected by bacterial strain, temperature, and salt and by their interactive effects, with strain F2365 being the most heat-resistant among all three strains tested. Salt added to salmon roe significantly increased the thermal resistance of the bacteria. For *L. monocytogenes* F2365, the z value of the bacterium in salmon roe was 5.99°C, and its heat resistance increased with the level of salt in a linear manner. The results of kinetic analysis and the models obtained in this study may be used by the seafood industry to develop proper thermal processes to eliminate *L. monocytogenes* in raw salmon roe and to ensure microbial safety and prevent foodborne illness.

Keywords: *Listeria monocytogenes*, thermal inactivation, salmon roe, salt effect

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44 1. Introduction

45 Caviars are lightly salted and preserved fish roe products with high nutritional and
46 economic values. While the most widely recognized and valued caviar is made from sturgeon,
47 salmon caviar is one of the most popular products because of its relative abundance, attractive
48 red color, and distinctive taste. The U.S. is one of the biggest producers of salmon roe and
49 exports 90% of the products to Asia and Europe (Bledsoe, Bledsoe, & Rasco, 2003). The U.S.
50 exported 11,738 metric tons of salmon caviar and roe, worth of \$163 million in 2011 (NOAA,
51 2011) and 15,307 metric tons, worth of \$255 million in 2013 (NOAA, 2014).

52 Grown in the ocean, fish may carry a variety of spoilage and pathogenic microorganisms,
53 such as *Vibrio parahaemolyticus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp.
54 (Novotny et al., 2004). While live fish flesh and eggs are sterile, the food processing
55 environment is not sterile, leading to cross-contamination in the products. *L. monocytogenes* is
56 frequently found in fish processing environment (Lunestad, Truong, & Lindstedt, 2013) and has
57 been found in various fish roe products (Jami et al., 2014). Himelbloom and Crapo (1998)
58 reported that aerobic plate counts (APCs) greater than 10^7 CFU/g and coliform counts higher
59 than 10^3 CFU/g were detected in Alaska salmon caviar. Miya et al. (2010) reported that *L.*
60 *monocytogenes* was detected in up to 11.4% of cod roe (tarako) samples and 10.0% of salmon
61 roe (ikura) samples tested. Other pathogens, such as *V. parahaemolyticus*, *Salmonella* spp.,
62 *Staphylococcus aureus*, *E. coli*, *Shigella* spp., and *Clostridium perfringens*, were found in grey
63 mullet roe (Voidarou et al., 2011). According to Bledsoe, Bledsoe, & Rasco (2003), salmon roe
64 is low in acidity (pH 5.2-6.7) and high in water activity (0.96-0.98), and is capable of supporting

the growth of microorganisms. Therefore, fish roe products are a potential vehicle for transmission of foodborne pathogens such as *L. monocytogenes*.

L. monocytogenes is a Gram-positive, non-spore forming bacterium that is ubiquitously distributed in the environment. It has been isolated from water, soil, food products, and the intestinal tracts of humans and animals (ICMSF, 1996). This bacterium is capable of growing at adverse environmental conditions, such as low temperature (4°C), high salt concentration (>10%), and low pH (McClure et al., 1989). Consumption of food contaminated with *L. monocytogenes* can lead to listeriosis, which is rare but with a relatively high mortality rate. The Centers for Disease Control and Prevention (CDC) estimated that *L. monocytogenes* causes approximately 1,600 illnesses, 1,500 hospitalizations, and 260 deaths annually in the U.S. (Scallan et al., 2011). To reduce foodborne listeriosis and protect public health, a “zero-tolerance” policy for this pathogen in cooked and RTE foods has been implemented in the U.S. (USDA-FSIS, 2003).

While fish roe products can be eaten raw, they may be inadvertently contaminated with *L. monocytogenes*. Once they are contaminated, an intervention process is needed to inactivate the pathogen. While some emerging nonthermal technologies such as pulsed electric field (Gudmundsson and Hafsteinsson, 2001) have been reported for use to control *L. monocytogenes* in fish roe, the scale-up feasibility and efficacy of these technologies remain uncertain and need further examination. Thermal processing remains a method of choice as an effective intervention strategy to inactivate pathogenic and spoilage microorganisms in foods, including fish roe products.

Thermal treatment has been found to be suitable for inactivation of *L. monocytogenes* and *Listeria innocua* (an surrogate) in fish roe (Al-Holy et al., 2004a; 2004b; Miettinen, Arvola, & Wirtanen, 2005) and *L. monocytogenes* in rainbow trout roe (Mittinen et al., 2005) containing 2.5% of salt. Generally, fish roe products contain 3.0 to 4.0% of salt (Craig and Powrie, 1988). The species of salmon and the condition and degree of maturity of the roe can affect the uptake of salt in fish roe (Huang, 2001). According to Jorgensen, Stephens, & Knochel (1995), osmotic adaption by exposure to NaCl significantly increases the thermotolerance of *L. monocytogenes* in modified tryptic phosphate broth and minced beef. However, little information is available concerning the effect of different concentrations of salt on the thermal resistance of *L. monocytogenes* in salmon roe. Therefore, the main objective of this study was to investigate the effect of salt on the thermal resistance of three main serotypes of *L. monocytogenes* in salmon roe. The results obtained from this research may help the food industry to design effective thermal treatments for controlling *L. monocytogenes* in salmon roe products.

2. Materials and Methods

2.1. Bacterial strains and preparation of inoculum

Three rifampicin-resistant (100 mg/L) *L. monocytogenes* strains, including *L. monocytogenes* serotype 4b (F2365), *L. monocytogenes* serotype 1/2b (F4260), and *L. monocytogenes* serotype 1/2a (V7), were used in this study. The antibiotic-resistant strains of *L. monocytogenes* were obtained from the culture collection of the Eastern Regional Research Center (ERRC) of the USDA Agricultural Research Service (ARS) located in Wyndmoor, PA (Fang, Liu, & Huang, 2013; Fang and Huang, 2014; Li et al. 2016), and were used to

differentiate the inoculated *L. monocytogenes* from numerous background bacteria in the salmon roe samples. The cultures were prepared by streaking each strain of the overnight culture onto tryptic soy agar (TSA, BD/Difco Laboratories, Sparks, MD) plates supplemented with 100 mg/L rifampicin (TSA/R, Sigma, R 3501-5G, Sigma-Aldrich Co., MO). To maintain the viability of the cells, the rifampicin-resistant *L. monocytogenes* cultures were regularly propagated and maintained on TSA/R plates and stored at 4°C.

One day prior to an experiment, a loopful of each strain was individually inoculated into 10 ml brain heart infusion broth (BHI broth, BD/Difco Laboratories) supplemented with 100 mg/L rifampicin and incubated at 37°C on an orbital shaker with mild agitation (~100 rpm) for approximately 18-20 h. The cultures were harvested by centrifugation (2400×g) at 4°C for 15 min, washed once with 10 ml 0.1% peptone water (PW, BD /Difco Laboratories), re-centrifuged, and re-suspended in 5 ml 0.1% PW. Each culture was used as the working culture directly, containing a final concentration of approximately 10⁹ CFU/ml of *L. monocytogenes*.

2.2. Sample preparation and inoculation

Unsalted fresh pink salmon roe was obtained from a seafood company in Alaska. The samples were divided into smaller bags (50 g) and frozen at -80°C. One night before experiment, a bag of salmon roe was transferred to a refrigerator (4°C) for thawing. The thawed salmon roe samples (1±0.02 g) were aseptically weighted into sterile filter bags (Whirl-Pak®, 207 ml, 95 mm × 180 mm × 0.08 mm, NASCO, Fort Atkinson, WI). Salmon roe with different salt concentrations was prepared by adding a different volume of a salt solution (25%) into the sample bags, with the final salt concentration of ca. 0, 1.5, 3 and 4.5%. Each sample bag was

individually inoculated with 100 µl of each strain of *L. monocytogenes* culture. The inoculated salmon roe was smashed by hand and gently mixed for at least 2 min, and then flattened with a round bottle to a thin layer (< 0.2 mm). To ensure uniform heating, the sample bags were vacuumed to evacuate the internal air and then sealed when the internal pressure reached 2.0 kPa. Uninoculated samples were used as negative controls. The population of *L. monocytogenes* was ca. 7.0 – 8.5 log CFU/g in the inoculated samples.

2.3. Thermal inactivation

The inoculated samples bags were subjected to submersion heating in a circulating water bath (Neslab RTE17, Thermo Fisher Scientific, Newington, NH) maintained at 57.5, 60, 62.5, or 65°C. The samples were fully submerged in hot water during heating. The come-up time, or the time needed to reach the treatment temperatures, was approximately 6 s and was excluded from the heating time. Duplicate samples were removed from the water bath at different time intervals and immediately plunged into an iced-water bath. The sampling time was adjusted according to the heating temperature. Each temperature and salt combination was repeated at least twice on separate trials.

2.4. Enumeration of *L. monocytogenes*

Heat-treated and control samples were aseptically opened, added with 9 ml of PW, and homogenized for 2 min on each side at the maximum speed in a stomacher (Model BagMixer 100W, Interscience Co., France). After homogenization, a small volume (0.1 ml) of the liquid

portion of the samples was withdrawn and plated, either directly or after serial dilutions, onto TSA/R plates in duplicate. When thermal treatment resulted in low bacterial counts, the surviving cells were recovered by plating 1 ml of undiluted sample onto TSA/R plates (3x). The plates were kept at room temperature for 2 h to allow the heat-injured cells to resuscitate, and then incubated at 37°C for 48h. The background microflora in the samples was suppressed by rifampicin in TSA/R plates and only rifampicin-resistant *L. monocytogenes* cells were recovered after incubation. The colonies were counted and recorded as log CFU/g.

2.5. Calculation of *D*- and *z*-values

The survival curves of *L. monocytogenes* were obtained by plotting the log counts against the heat treatment times at each temperature. The lines of best fit for survivor plots were determined by linear regression with Microsoft Excel (Microsoft Corp., Redmond, WA) and the negative reciprocals of the slopes were calculated as the decimal reduction times (*D*-values). The logarithms of *D* were also plotted against temperature to determine the *z*-value of the bacteria by calculating the inverse of the slope.

2.6. Statistical analysis

The effect of bacterial strains, temperature, and salt concentration on thermal resistance (log *D*-values) of *L. monocytogenes* in salmon roe was analyzed using analysis of variance (ANOVA) of SAS version 9.3 (SAS Institute, Cary, NC) for the means of log *D*-values to assess whether each treatment or their interaction has a significant effect on thermal resistance. The

hypothesis (H_0) was that these factors and their interactions had not significant effect on thermal resistance of the bacterium. A p value of < 0.05 for each treatment or their interaction indicates a significant effect. If a significant effect was observed for a treatment, the t-tests (LSD) procedures were used for pairwise comparison. A p value of < 0.05 indicates a significant difference.

Linear regression was used to explore the effect of temperature and salt on thermal resistance of the most heat-resistant strain of *L. monocytogenes* using Eq. (1). The linear regression was performed using Proc REG of SAS.

$$(1)$$

2.7 Total plate counts, pH, and water activity

The total plate counts (TPC) were determined by plating the control samples (without any treatment) onto TSA plates, which were incubated under 37°C overnight. For measuring the pH of the salmon roe, a sample (5 g) was first stomached for 2 min and then loaded into a plastic centrifuge tube. The pH of salmon roe was determined in the centrifuge tube using a Corning pH meter (Model 430, Corning Inc., New York). The water activity (A_w) of the samples was measured by using a water activity meter (Dew Point Water Activity Meter Series 4, AquaLab, Pullman, WA). Both pH and A_w were measured at room temperature. The determination of TPC, pH, and A_w was performed in triplicate.

3. Result and Discussion

3.1 Total aerobic count, pH and A_w of salmon roe

The total aerobic count of background microflora in salmon roe was 2.3 log CFU/g (Table 1). No *L. monocytogenes* was found in the samples by direct plating. The pH and A_w of raw salmon roe were 6.15 and 0.987, respectively, whereas the A_w values were 0.982-0.953 with 1.5-4.5% salt added (Table 1).

3.2 Thermal resistance of *L. monocytogenes* in salmon roe

When the salmon roe samples were heated under a constant temperature between 57.5 and 65°C, the log counts of *L. monocytogenes* declined in a linear manner with heating time (Fig. 1), suggesting that the inactivation of this microorganism in salmon roe followed a 1st order kinetics. The D-values of *L. monocytogenes* varied with heating temperature, bacterial strains, and the amount of salt added to the samples (Table 2).

The results of ANOVA (Table 3) showed that the thermal resistance (log D) of *L. monocytogenes* in salmon roe was significantly affected by bacterial strain, temperature, salt, and interactions between these individual factors ($R^2 = 0.994$, $p < 0.0001$). The p values of the individual effect of bacterial strain, temperature, and salt, and the interactive effect between strain and temperature (Strain×Temp) were all < 0.0001 . For the interactive effects between temperature and salt (Temp×Salt) and strain and salt (Strain×Salt), the p values were 0.0420 and 0.0186, and both are < 0.05 . The results of ANOVA shown in Table 3 clearly suggest that the survival of *L. monocytogenes* in salmon roe is highly dependent on the strain of the bacterium, temperature applied, and the amount of salt added to salmon roe. Therefore, the hypothesis (H_0)

that bacterial strains, temperature, and salt concentration had no significant effect on thermal resistance (log D-values) of *L. monocytogenes* was, therefore, rejected.

The LSD tests were used to further compare the effect of different treatments on log D-values of *L. monocytogenes*. Shown in Table 4, *L. monocytogenes* F2365 was the most heat-resistant, and V7 was the least heat-resistant among the three strains tested (Table 4), suggesting that thermal processes for salmon roe should be designed to target *L. monocytogenes* F2365 among the three strains used in this study. The LSD tests confirmed that salt significantly increased the thermal resistance of *L. monocytogenes* in salmon roe.

In Table 2, the D-values of *L. monocytogenes* F4260 were higher than those of *L. monocytogenes* F2365 at 57.5°C. However, the D-values of this strain were lower than those of *L. monocytogenes* F2365 at 62.5 and 65°C, suggesting that *L. monocytogenes* F4260 was more sensitive to heat than *L. monocytogenes* F2365 at higher temperatures. Since *L. monocytogenes* F2365 was the most heat-resistant strain among the three strains, the thermal resistance of this strain was further analyzed by linear regression to examine the interactive effect of temperature and salt on log D-values during heating. While the salt added to salmon roe significantly affected the thermal resistance of *L. monocytogenes* when all three strains were evaluated together, further analyses showed that the salt alone did not significantly affect the thermal resistance of *L. monocytogenes* F2365 ($P > 0.05$). The effect of salt on thermal resistance of *L. monocytogenes* F2365 was manifested in the interactive effect between heating temperature and salt, and can be expressed using Eq. (2), with regression parameters listed in Table 5.

$$(2)$$

According to Eq. (2) and the parameters listed in Table 5, the effect of temperature on thermal resistance of *L. monocytogenes* F2365 can be expressed using Eq. (3). Based on this equation, the z value of *L. monocytogenes* F2365 without salt is 5.99°C, which is the pure effect of temperature on thermal resistance. According to Eq. (3), adding salt to salmon roe decreases the sensitivity of the bacterium to heat, making it more heat-resistant.

$$(3)$$

In the study reported by Al-Holy et al. (2004a), the D-values of *L. innocua* in salmon caviar (2.5% salt) were determined as 2.97, 0.77, and 0.40 min at 60, 63, and 65°C, respectively. According to Eq. (2), the D values of *L. monocytogenes* F2365 in salmon roe with 2.5% is estimated as 3.12, 1.03, and 0.49 min at 60, 63, and 65°C, respectively. In a study reported by Miettinen, Arvola & Wirtanen (2005), the thermal resistance of a mixture of four *L. monocytogenes* strains was evaluated in rainbow trout roe (2.5% salt, w/w). The D-values of *L. monocytogenes* were determined as 1.60 and 0.44 min at 60 and 63°C, respectively. Apparently, the thermal resistance of *L. monocytogenes* F2365 was numerically higher than that reported by Al-Holy et al. (2004a) and Miettinen, Arvola, & Wirtanen (2005). The difference in thermal resistance between different studies may be due to the difference in the bacterial strains and the substrates. The results of this study also pointed out the necessity to identify and use the most heat resistant strain of the bacterium as the target for designing thermal processes.

4. Conclusion

L. monocytogenes in salmon roe products is a food safety concern. Once contamination occurs, it is necessary to render salmon roe free of *L. monocytogenes* for human consumption. Thermal processing can be used to eliminate *L. monocytogenes* in salmon roe. This study investigated the effect of salt and temperature on survival of three strains of *L. monocytogenes* (F2365, F4260, and V7) during heating. The results showed that the thermal resistance of *L. monocytogenes* in salmon roe was significantly affected by bacterial strain, temperature, and salt concentration and by their interactive effects, with *L. monocytogenes* F2365 being the most heat-resistant among all three tested. Adding salt to salmon roe increased the thermal resistance of *L. monocytogenes*. The results of kinetic analysis and the models obtained in this study may be used by the seafood industry to develop proper thermal processes to eliminate *L. monocytogenes* in raw salmon roe to ensure microbial safety and prevent foodborne illness.

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330 **Table 1.** Total plate count, pH, and water activity of salmon roe.

Parameters	Values (mean $\pm$ SD [*])
Total plate counts	2.3 $\pm$ 0.3 log CFU/g
pH	6.15 $\pm$ 0.03
Water activity (A_w)	0.987 $\pm$ 0.001 (0.0% salt)
	0.982 $\pm$ 0.003 (1.5% salt)
	0.958 $\pm$ 0.002 (3.0% salt)
	0.953 $\pm$ 0.002 (4.5% salt)

331 ^{*} SD: one standard deviation.

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333 Table 2. D-values (min) of *L. monocytogenes* in unsalted and salted salmon roe between 57.5 and

334 65.0°C

Temp (°C)	Salt concentration (%)			
	0	1.5	3.0	4.5
<i>L. monocytogenes</i> F2365				
57.5	3.42±0.11	5.55±0.49	9.48±1.63	12.09±1.66
60.0	1.34±0.24	2.43±0.05	4.15±0.36	5.55±0.16
62.5	0.48±0.04	1.00±0.09	1.53±0.37	2.40±0.03
65.0	0.14±0.01	0.35±0.02	0.82±0.01	0.79±0.12
<i>L. monocytogenes</i> F4260				
57.5	6.67±0.53	10.74±1.04	16.78±1.84	19.67±1.58
60.0	1.39±0.08	2.79±0.44	4.45±0.38	6.77±0.64
62.5	0.38±0.02	0.65±0.06	0.98±0.23	1.42±0.04
65.0	0.10±0.01	0.17±0.01	0.36±0.06	0.50±0.03
<i>L. monocytogenes</i> V7				
57.5	3.96±0.04	8.95±0.32	10.73±1.19	17.13±1.73
60.0	1.37±0.01	2.30±0.04	4.14±0.07	5.03±0.19
62.5	0.30±0.03	0.50±0.02	0.72±0.02	1.21±0.23
65.0	0.11±0.01	0.20±0.01	0.21±0.04	0.56±0.12

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337 Table 3. Results of ANOVA for evaluating the effect of bacterial strain, temperature, and salt

338 concentration on thermal resistance (log D-values) of *L. monocytogenes* in salmon roe.

Source	DF	Sum of Squares	Mean square	F value	Pr > F
Model	29	38.09	1.31	348.5	< 0.0001
Error	66	0.25	0.0038		
Corrected total	95	38.34			
	R ²	Coeff Var	Root MSE	log D Mean	
	0.994	31.28	0.061	0.196	
Source	DF	Anova SS	Mean Square	F value	Pr > F
	2	0.219	0.110	29.1	< 0.0001
Temp.	3	31.57	10.52	2791.5	< 0.0001
Salt	3	5.39	1.80	477.0	< 0.0001
Temp×Salt	9	0.071	0.0079	2.10	0.0420
Strain×Temp	6	0.777	0.130	34.37	< 0.0001
Strain×Salt	6	0.062	0.010	2.76	0.0186

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341 Table 4. Results of pairwise comparison (LSD test) of the means of log D-values of *L.*342 *monocytogenes* as affected by bacterial strain, temperature, and salt concentration.

Effect of Bacterial Strain			
t Grouping	Mean	N	
A*	0.245	32	F2365
B	0.213	32	F4260
C	0.131	32	V7
Effect of Temperature			
t Grouping	Mean	N	Temperature
A	0.959	24	57.5
B	0.478	24	60.0
C	-0.094	24	62.5
D	-0.558	24	65.0
Effect of Salt			
t Grouping	Mean	N	Salt
A	0.485	24	4.5
B	0.325	24	3.0
C	0.124	24	1.5
D	-0.149	24	0.0

343 * Means in column for each effect with different letters are significantly different ($P < 0.05$). N:

344 number of observations for each treatment.

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 346 Table 5. Results of linear regression analysis of the effect of temperature and salt on log D-
 347 values of *L. monocytogenes* F2365.

Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Model	2	8.55	4.28	632	< 0.0001
Error	29	0.20	0.01		
Corrected Total	31	8.75			
Root MSE		0.082	R ²	0.978	
Dependent Mean		0.245	Adjusted R ²	0.976	
Coeff Var		33.6			
Parameter Estimates					
Variable	DF	Parameter Estimate	Standard Error	t value	Pr > t
Intercept (a)	1	10.15	0.319	31.84	< 0.0001
Temp (b)	1	-0.167	0.005	-32.10	< 0.0001
Temp×Salt (d)	1	2.43e-3	1.41e-4	17.22	< 0.0001

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Figure 1. Representative survival curves of the *Listeria monocytogenes* F4260 in salmon roe (3% salt) subjected to different heating different temperatures.

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