Predicting the effect of salt on heat tolerance of Listeria monocytogenes in meat and fish products

Hansen, Tina Beck; Abdalas, Somaya; Al-Hilali, Iman; Hansen, Lisbeth Truelstrup

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Title: Predicting the effect of salt on heat tolerance of *Listeria monocytogenes* in meat and fish products

Authors: Tina Beck Hansen, Somaya Abdalas, Iman Al-Hilali, Lisbeth Truelstrup Hansen

Affiliation: The National Food Institute, Technical University of Denmark, Division for Microbiology and Production, Kemitorvet, Building 202, DK-2800 Kgs. Lyngby, Denmark

Corresponding author: Tina Beck Hansen, The National Food Institute, Technical University of Denmark, Division for Microbiology and Production, Kemitorvet, Building 202, DK-2800 Kgs. Lyngby, Denmark, email: tibha@food.dtu.dk

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Abstract

*Listeria monocytogenes* is a potentially fatal foodborne pathogen that can be found in various ready-to-eat (RTE) products. It tolerates adverse conditions such as high salt concentrations and refrigerated storage, thus, the elimination of the pathogen in food processing often relies on heat processing. The objective of this study was to create a model to predict the effect of salt on heat tolerance of *L. monocytogenes* in meat and seafood products during heat treatments conducted at 57 to 65 °C to reduce numbers by ≥3 log₁₀ cycles. Salt concentrations, up to 6% in the water phase (WPS%), were applied to cover a variety of lightly salted RTE meat and seafood products. The experimental work involved samples of ground pork tenderloin, ground chicken breast fillet and skinned, ground salmon fillet adjusted to different WPS% *i.e.*, 3.6 and 5.2 WPS% for pork samples, 2.0, 3.0, 3.5 and 6.0 WPS% for chicken samples and 3.0 and 6.0 WPS% for salmon samples. All samples were inoculated with late-stationary phase *L. monocytogenes* cultures. For pork samples, a two-strain mixture of a pork isolate (MS22254) and an environmental isolate (MS22246) was applied. For chicken and salmon samples, a seafood isolate (MS22258) and isolate MS22246 was applied as single cultures. Samples were vacuum-packed in sterile bags, immersed in water bath, and held at constant temperatures of 57, 60 and 65 °C for pork samples and 58, 61 and 62.5 °C for chicken and salmon samples.

For survivor curves, where at least 3 log₁₀-reduction were obtained, heat tolerance was expressed as decimal reduction times, D-values. D-values were observed to increase with increasing WPS%. The effect of salt on heat tolerance of *L. monocytogenes* was defined as the relative increase (*RI*-value) in D-value obtained when salt had been added to the food. The effect of WPS% on *RI*-values was independent of heating temperatures, foods and strains. For secondary modelling, *RI*-values were transformed using the natural logarithm, ln(*RI*) and fitted to a linear model as a function of WPS%. Model validation, with 56 independent values collected from the scientific literature, resulted in bias and accuracy factors of 0.89 and 1.26, respectively, suggesting acceptable performance with tendency to slightly under-predict. The developed predictive model can be used to guide the design of heat processes for manufacturers of lightly preserved and mildly processed meat and seafood products requiring more than 3 log₁₀ reduction of *L. monocytogenes* to ensure safety.
Listeria monocytogenes is a well-known food-borne human pathogen. The widespread distribution of the organism in nature and livestock makes the presence of *L. monocytogenes* in raw meats and ready-to-eat (RTE) foods difficult to avoid (EFSA BIOHAZ Panel, 2018). Therefore, contamination of RTE meat and seafood products with *L. monocytogenes* is known to result in illnesses and poses a special threat to the public health due to the ability of the bacterium to grow at refrigeration temperatures (EFSA, 2013; NicAogáin and O’Byrne, 2016). Listeriosis, the illnesses caused by the pathogen, can have severe outcomes such as meningitis, miscarriages and deaths, especially for persons belonging to the risk groups (YOPI, young, old, pregnant and immune compromised individuals).

Meat and seafood products support growth of *L. monocytogenes* due to their composition providing the essential nutrients for the pathogens to grow (Buchanan et al., 2017). Usually, the risk of getting ill from food-borne pathogens in food products can be reduced by application of preservation methods including combinations of heat treatment and salt addition. Heat inactivation studies have shown diverging behaviour of *L. monocytogenes* in the presence of salt with elevated salt concentrations in the heating menstruum resulting in enhanced heat tolerance (Juneja and Eblen, 1999; van Asselt and Zwietering, 2006; Yen et al., 1991). This cross-protection phenomenon is explained by the salt addition resulting in an osmotically induced thermotolerance, making it interesting to quantitatively study the behaviour of *L. monocytogenes* subjected to such combined treatments (Chen, 2017).

The existing evidence of increasing heat tolerance of *L. monocytogenes* with increasing salt levels has been studied extensively in broth-model systems (Cole et al., 1993; Jørgensen et al., 1995; Juneja and Eblen, 1999) which may not be comparable, at the quantitative level, to solid food products due to differences in physical, structural and chemical properties. For solid foods, sporadic reports have been published (Farber, 1989; Farber et al., 1989; Jørgensen et al., 1995; Mackey et al., 1990; Schoeni et al., 1991) and more recently, predictive models describing the combined effect of temperature and NaCl on heat tolerance of *L. monocytogenes* in beef (Juneja et al., 2013) and in salmon roe (Li et al., 2017) have been developed. It is, however, uncertain whether these models can be applied to other meat and seafood products. Therefore, new thermal death time data for solid food-model systems are needed to attempt the development of new generic models, in order to be able to quantify the reported salt-effect on the heat tolerance of this pathogen in a broader selection of meat and seafood products.

In addition, the food processing sector is moving toward even milder processing conditions in response to the consumer perceptions that the texture, taste, vitamins and minerals might be denatured or destroyed during heating processes at high temperatures. Therefore, lower heating temperatures are preferred to
ensure tender, juicy products and preservation of the taste as well as higher vitamin content in the heated products (Lešková et al., 2006; Nikmaram et al., 2011).

The aim of this study was to model the effect of salt on the heat tolerance of \textit{L. monocytogenes} in pork, chicken and salmon during mild heat treatments at constant temperatures ranging between 57 and 65 °C. Various salt concentrations, 2.0, 3.0, 3.6, 5.2 and 6.0% in the water phase, were applied in order to cover a variety of lightly salted RTE meat and seafood products. It was hypothesized that the salt-induced effect on the heat tolerance of \textit{L. monocytogenes} only depended on water phase salt (WPS%) in the products and not on the types of food nor the heating temperatures. A generic model, predicting the factor by which heat tolerance increased with increasing WPS%, was developed.

2. Materials and methods

2.1 Strains and inocula

2.1.1 Selection of \textit{Listeria monocytogenes} strains

Six strains of \textit{L. monocytogenes} were selected from the European Union Reference Laboratory (EURL) reference strain collection (Bégot et al., 1997; Guillier et al., 2013) at the National Food Institute at the Technical University of Denmark (DTU). As the list did not include prior information on the heat tolerance of the strains, selection was primarily based on their origin and hence meat, seafood and environmental isolates were represented in the study. When more strains per origin were available, the ones exhibiting the highest salt tolerance were selected based on maximum growth rates at a water activity of 0.95 as indicated by Guillier et al. (2013). To assist the final selection, two strains from each origin were picked for determination of heat tolerance in Brain Heart Infusion (BHI) broth (Oxoid, UK).

The two strains isolated from seafood products (MS22258 [EURL no. 12MOB100LM] and MS22261 [12MOB103LM]), the two strains isolated from industrial environments (MS22246 [12MOB048ML] and MS22248 [12MOB050LM]) and the two strains isolated from pork products (MS22243 [12MOB045LM] and MS22254 [12MOB089LM]) were maintained at -80 °C in 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd, Heywood, UK). Each strain was streaked on Tryptone Soy Agar (TSA) plates with 5% (v/v) sheep blood (Oxoid, UK) and incubated at room temperature (20 – 25 °C) for 3 d. Subsequently, colony material from 5 to 10 colonies of each strain was added to separate 15-ml centrifuge tubes filled with 10 ml BHI broth. The culture suspensions were incubated for 24 h without shaking at 37 °C. New batches of the cultures were prepared by diluting the 24-h culture 1:100 in 0.9% (w/v) saline and transferring a volume of 0.5 ml to 50 ml BHI broth in 50-ml blue-cap flasks. These subcultures were incubated at 37 °C for 24 h. Average cell concentrations of 9.1 ± 0.2 log_{10} cfu/ml were obtained for all
strains as confirmed by serially diluting samples followed by drop-plating 3 x 10 µl on TSA and incubation at 37 °C for 24 – 33 h.

A volume of 1 ml of each of the cultures was inoculated into separate conical flasks filled with 100 ml BHI broth that had been pre-heated to 60 °C in a shaken water bath (Julabo SW 22, Buch & Holm, Denmark). The samples were heat-treated for 15 min at 60 °C. One uninoculated flask was dedicated to temperature measurements with a standard precision thermometer (Buch & Holm, Denmark) and the temperature was read six times during heat exposure. For each strain, a total of nine samples were collected at time intervals of 1 – 3 min. For enumeration of survivors, two samples of 100 µl were taken, one plated directly on TSA and the other diluted 10-fold in BHI before plating 100 µl on TSA and incubating at 37 °C for 4 d. BHI broth was used as dilution medium as nutrient-rich media are expected to give an effective recovery of heat-injured cells (Busch and Donelly, 1992; Hansen, 1996). Survivor curves were established and the most heat tolerant strain for each of the origins; meat, seafood and industrial environment, were selected for further experiments.

2.1.2 Preparation of standardized frozen cultures

For the selected strains (MS22246, MS22254, MS22258), stocks of frozen cultures were produced by using subcultures prepared as described in section 2.1.1. The stocks were made by transferring 500 µl from each culture to 50 ml BHI broth, mixing and adding 21 ml glycerol (Fisher Chemical, USA) and mixing again, thoroughly, to avoid phase separation. These mixtures were distributed in aliquots of 1 ml in 1.5-ml microtubes (Sarstedt Ag & Co. Kg, Germany) and kept at -20 °C. The concentration of each strain was measured after thawing at room temperature to be approx. 7 log_{10} cfu/ml by performing serial 10-fold dilutions in BHI, drop-plating of 3 x 10 µl of suitable dilutions on TSA followed by incubation at 37 °C for 24 – 33 h.

2.1.3 Preparation of inocula for heat inactivation trials

Heat inactivation trials were conducted for single strains as well as for cocktails of two strains, as described below. Prior to each heat inactivation trial, cryotubes with 1 ml of standardized frozen cultures of the strains were thawed at room temperature and inoculated into 100-ml bottles containing 40 ml BHI broth to obtain an initial concentration of approx. 10^5 cfu/ml. The culture suspensions were incubated at 37 °C for at least 30 h to obtain late-stationary phase cells. Preliminary tests showed that the selected strains entered the stationary growth phase after 8 to 11 hours using the described procedure (data not shown).
Five ml of each stationary phase culture were transferred into separate 15-ml centrifuge tubes. Cells were harvested by centrifugation at 3,800 rpm for 10 min at 20 °C (IECCentra-8R centrifuge, Buch & Holm, Denmark). The supernatants were discarded, and the pellets washed once with 5 – 10 ml 0.9% saline by mixing with a vortex mixer (Bie & Berntsen A/S, Denmark), re-centrifuged, and finally re-suspended in 5 ml 0.9% saline. For trials where single strains were tested, these culture suspensions were used directly. For trials, where cocktails of two strains were tested, culture suspensions were mixed 1:1 before use.

2.2 Samples and inoculation

2.2.1 Preparation of pork samples

Fresh raw pork tenderloin was obtained from a local retail store in Copenhagen. The pork was ground by using a meat grinder (Kenwood Chef type KVC30, Kenwood Ltd., UK) and separated into portions of 100 g in 4-L-freezer bags and frozen at -20 °C. One day prior to heating trials, bags of 100 g frozen pork were transferred to room temperature (20 °C) for thawing. After thawing, the pork samples with different salt concentrations were prepared by adding calculated amounts of crystalline NaCl (Emsure®, Merck KGaA, Germany) to the meat. The added salt was then blended into the meat by pressing the bag against the laboratory bench several times and massaging it by hand. To ensure homogenous distribution of the salt, all samples rested at 5 °C until the following day. Final water-phase-salt (WPS) concentrations were 0.2 (no added salt), 3.6 and 5.2% (Table 1). On this day, meat samples of 10 ± 0.5 g were weighed into separate small vacuum bags (NEN 40 HOB/LLDPE 75, Amcore Flexibles, Denmark) (8.5 x 4.5 cm) and used in the heat inactivation trial (see section 2.3).

2.2.2 Preparation of chicken and salmon samples

Chicken breast fillets without skin and salmon fillets with skin were purchased from a local retail store. Before each heat inactivation trial, chicken or salmon without skin were ground in a meat grinder and separated into portions of 100 g in 4-L-freezer bags for preparation of samples with different salt concentrations. The skin was removed from the salmon before mincing. Calculated amounts of salt were added to the 100-g-portions of ground chicken and ground salmon to result in final WPS of 2, 3 and 6% (Table 1). After addition of salt, the 100-g portions were blended thoroughly by hand for 1 min and rested for 20 min at room temperature and then blended again by hand for another 1 min. After blending, samples of 10 ± 0.5 g were weighed into 12 x 10 cm vacuum bags (NEN40/LLDPE 75; Amore Flexibles) and stored at 5 °C until the following day.
Table 1

Water-phase-salt contents (WPS%) in pork, chicken and salmon applied in the present study.

<table>
<thead>
<tr>
<th>Food</th>
<th>Mean % moisture\textsuperscript{a} (SD)</th>
<th>Salt\textsuperscript{b} per 100 ± 0.5 g food (g ± 0.01 g)</th>
<th>Mean WPS%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground pork tenderloin</td>
<td>74 (0.6)</td>
<td>0.17\textsuperscript{c}</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.77</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.02</td>
<td>5.2</td>
</tr>
<tr>
<td>Ground chicken breast fillet</td>
<td>75 (0.3)</td>
<td>0.20\textsuperscript{c}</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.54</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.32</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.72</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.78</td>
<td>6.0</td>
</tr>
<tr>
<td>Ground, skinned salmon fillet</td>
<td>68\textsuperscript{d}</td>
<td>0.05\textsuperscript{c}</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.10</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.32</td>
<td>6.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Estimated from the declared nutritional composition
\textsuperscript{b}Denotes the total salt content from the endogenous and added salt in the products
\textsuperscript{c}Endogenous level as declared
\textsuperscript{d}Not possible to estimate SD, as only one batch was applied

2.2.3 Inoculation of food samples

Each sample was individually inoculated with 1% (v/w) of the washed stationary culture inoculum by injecting 100 µl into the centre of the sample. The inoculated sample was blended thoroughly by hand for 30 s, and then flattened to a thin layer (max. 4 mm). To ensure uniform heating, samples were vacuum-packaged (Multivac Ltd., Bibby sterilin Ltd., UK) to remove the internal air. Enumeration, performed as described below in section 2.4, showed that the initial \textit{L. monocytogenes} concentration was 7.1 ± 0.2 log\textsubscript{10} cfu/g in the inoculated food samples allowing for the quantification of up to 5 log\textsubscript{10} reduction during the heat treatment.
2.3 Heat treatment

The bags with inoculated samples were immersed in a circulating water bath (Julabo SW 22, Buch & Holm, Denmark or Grant, VWR International, Denmark) maintained at 57.0, 60.0 and 65.0 °C for pork, and 58.0, 60.0, 61.0 and 62.5 °C for chicken and salmon samples. The water temperature was monitored continuously using a digital thermometer (Oregon Scientific, model no. SA880SSX, USA). Samples were fully submerged in water during heating. Depending on the specific trial, six to eight samples were removed from the water bath at different time intervals and immediately placed into a chilled-water bath (10 – 15 °C) to stop the exposure to heat. The maximum treatment times depended on heating temperature, food, WPS% and strain (Table S1). Five trials were conducted as single determinations and 34 trials were repeated at least twice in biologically independent experiments (Table 2).

2.4 Enumeration of survivors

All samples were analysed individually. The heated sample bag was aseptically cut open with a pair of scissors and the content transferred to a sterile filter stomacher bag (Seward stomacher®lab system, classic 400, BA6041/STR, Seward, UK or BagPage®, Breveté, France), followed by addition of 90 ml BHI broth and homogenization for 3 – 4 min using a stomacher (Stomacher 400, Seward Medical, UK). After homogenization, 100 µl of the liquid portion of the samples was spread-plated on Palcam *Listeria* selective agar medium (Oxoid, UK) either directly from the stomacher bag or from suitable 10-fold dilutions made in 0.9 ml BHI broth in 1.5-ml Eppendorf tubes. Agar plates were incubated at 30 – 37 °C for 4 d to allow maximum recovery of heat-injured cells. *L. monocytogenes* colonies, appearing as grey/green with a black halo around colonies, were counted and recorded as log₁₀ cfu/g.

2.5 Water phase salt and pH

2.5.1 Calculation of water-phase-salt

Water phase salt (WPS) was calculated using equation 1;

$$ WPS (\%) = 100 \times \frac{\text{weight of salt per 100 g}}{(100 \% - \% \text{ dry matter}) + \text{weight of salt per 100 g}} $$

(1)

Where the dry matter contents were estimated based on the products’ declared nutritional composition. Weight of salt was calculated as the sum of the products’ declared endogenous salt content plus the experimentally added salt.
The variation width of WPS% were approximated based on the known uncertainties of moisture, weighed salt and weighed meat (Table 1). The lowest value in the variation width was estimated using the mean moisture plus 2 x standard deviation (n-1) (SD), the weighed salt load minus uncertainty (0.01 g), the endogenous salt percentage and the weighed food plus uncertainty (0.5 g). The highest value in the variation width was then estimated using the mean moisture minus 2 x SD, the weighed salt load plus uncertainty (0.01 g), the endogenous salt percentage and the weighed food minus uncertainty (0.5 g).

2.5.2 Measurement of pH

For measurements of pH, samples of 5 g of each of three batches of ground pork tenderloin, chicken breast fillet and salmon fillet, respectively, were placed in 50-ml disposable plastic containers and diluted 1:4 with autoclaved and deionised water. The samples were homogenised by a magnetic stirrer for 5 – 10 min (Heidolph MR 3001K) before the pH was measured with a pH-meter (Bie & Berntsen A-S, 744 pH-meter, Metrohm, DK). Prior to pH measurements, calibrations were performed with buffers at pH 4.0 and 7.0 (Radiometer analytical sas, France).

2.6 Data analyses

2.6.1 Fitting pork data to primary inactivation models

Survivor curves from the biological replicates, obtained for each of the heating trials conducted at different temperatures (57, 60, 65 °C) in pork with different WPS% (0.2, 3.6 and 5.2%), were pooled and fitted to three microbial inactivation models; the log-linear model (equation 2) (Bigelow & Esty, 1920), the Geeraerd_shoulder model (equation 3) (Geeraerd et al., 2000) and Weibull model (equation 4) (Mafart et al., 2002) using GInaFit, an excel add-in developed by Geeraerd et al. (2005).

\[
\log_{10}(N) = \log_{10}(N_0) - \frac{k_{\text{max}} \times t}{\ln(10)}
\]  

(2)

\[
\log_{10}(N) = \log_{10}(N_0) - \frac{k_{\text{max}} \times t}{\ln(10)} + \log_{10} \left( \frac{e^{k_{\text{max}} \times s_l} \times (e^{k_{\text{max}} \times s_l} - 1)}{1 + \left( e^{k_{\text{max}} \times s_l} - 1 \right) \times (e^{-k_{\text{max}} \times t})} \right)
\]  

(3)

\[
\log_{10}(N) = \log_{10}(N_0) - \left( \frac{t}{\delta} \right)^p
\]  

(4)
\[ No \text{ is the initial concentration of } L. \text{ monocytogenes (cfu/g)} \text{ and } N \text{ (cfu/g) is the surviving concentration at time } t \text{ (min). The parameter } k_{\text{max}} \text{ is the specific inactivation rate (1/min) during the exponential decay, } S_l \text{ is the shoulder length (min), } \delta \text{ and } p \text{ are the time to the first decimal reduction (min) and the shape parameter, respectively.} \]

Akaike information criterion with a correlation for small datasets (AICc) was applied for evaluating whether the Geeraerd_shoulder (eq. 3) or the Weibull (eq. 4) models provided a better fit than the log-linear model (eq. 2). AICc was calculated as described by Burnham and Anderson (2002) using equation 5:

\[ AICc = n \times \ln(\text{RSS}) + 2 \times k + \frac{(2 \times k^2 + 2 \times k)}{(n - k - 1)} \] (5)

where \( n \) is the number of observations of each survivor curve, \( \text{RSS} \) is the residual sum of squares obtained when fitting each of the log-linear (equation 2), the Geeraerd_shoulder (equation 3) and the Weibull (equation 4) models, and \( k \) is the number of parameters, including the RSS, of the models.

The absolute value of the difference between AICc for two models were used for model comparison. For differences greater than 10, the model with the highest AICc was considered unlikely (Burnham and Anderson, 2002).

2.6.1 Decimal reduction times

All survivor curves, where more than 3 log10-reductions were obtained, were individually fitted to the log-linear model (eq. 2) and the decimal reduction times (D-values) were calculated using equation 6:

\[ D - \text{value} = \frac{\ln(10)}{k_{\text{max}}} \] (6)

with \( k_{\text{max}} \) being the inactivation rate (1/min) estimated when fitting the survivor curves with GlNaFit.

2.6.2 Salt-induced heat tolerance

The salt-induced heat tolerance of \( L. \text{ monocytogenes} \) was defined as the relative change \( (RI)-\text{value} \) in heat tolerance that occurred with the changing content of salt in the foods \( i.e. \), the \( RI \)-value represent the factor by which the D-value increased with addition of salt. For each combination of food, strain, heating temperature and WPS%, the \( RI \)-value was estimated by dividing the D-value obtained, when salt was added, with the average D-value obtained without addition of salt (only endogenous salt) (eq. 7).

\[ RI(T_i) = \frac{D_{\text{salt}(T_i)}}{D_{\text{no salt}(T_i)}} \] (7)
with $D_{\text{salt}}$ and $D_{\text{no salt}}$ being the D-values with and without salt added, respectively, and estimated at the $i$th heating temperature, $T_i$.

2.6.3 Secondary modelling

All $RI$-values, $\ln(RI)$ and $\sqrt{RI}$, independent of foods, strains and heating temperatures, were plotted against WPS% to establish whether a transformation was required to stabilize the variance of the response variable. Each plot was fitted to a straight line using the regression function in Microsoft Excel (Microsoft Corporation, USA) and the transformation resulting in the most random residual plot was used for further analyses and secondary modelling.

The effect of food, heating temperature and strain on the salt-induced heat tolerance was investigated by linear regression performed in Microsoft Excel which estimated the average as well as the lower and upper confidence bands (CI95%) of the slope. When two or more CI95% overlapped they were considered as statistically non-significant from each other. For all non-significant effects, data were pooled and used for final secondary modelling of the effect of WPS% on the salt-induced heat tolerance of *L. monocytogenes*.

The following equation

$$\ln(RI) = a \times \text{WPS}\% + b$$

was applied for estimation of the CI95% of the slope, $a$ (1/WPS%) and the intercept, $b$.

2.6.4 Model validation

The final secondary model, which had been derived from the experimental work, was validated using independent data on heat tolerance for *L. monocytogenes* in salmon roe (Li et al., 2017), pork slurry (Lihono et al., 2003) and minced beef (Jørgensen et al., 1995; Juneja et al., 2013). $RI$-values were calculated as described in section 2.6.2. When values for WPS% of the heated foods were not available from the studies, values were calculated using eq. 2 based on the information given in the materials and methods sections of the articles.

Graphical illustration as well as bias ($B_f$) and accuracy factors ($A_f$) were applied for the evaluation. $B_f$ and $A_f$ were calculated in accordance to Ross (1996) using $RI$-values as the response variable.

$$B_f = 10^{\frac{\sum \log_{10}(\text{predicted } RI/\text{observed } RI)}{n}}$$

$$A_f = 10^{\frac{\sum \| \log_{10}(\text{predicted } RI/\text{observed } RI) \|}{n}}$$
with \( n \) as the number of observations.

3. Results

3.1 Selection of strains for food trials

Survivor curves for the six selected \( L. \) monocytogenes strains from the EURL list, heated in BHI at 60 \( \pm \) 0.5 °C, resulted in D-values ranging from 0.8 min to 5.5 min (Figure 1). Isolates from industrial environments, MS22246 and MS22248 exhibited the two highest \( D_{60} \)-values of 5.5 and 3.5 min, respectively. For the pork isolate, MS22254, the \( D_{60} \)-value was 3.0 min whereas \( D_{60} = 0.8 \) min was observed for the second pork isolate, MS22243. The two strains with seafood origin, MS22258 and MS22261, showed \( D_{60} \)-values of 2.5 and 2.2 min, respectively. The most heat tolerant strain from each origin \( i.e. \), MS22246 from industrial environments, MS22254 from meats and MS22258 from seafood, were selected for further studies in foods. Altogether, these strains represented genoserotypes II and IV and serotypes 1/2a and 4b (Guillier et al., 2013).

![Figure 1. Survivor curves of six Listeria monocytogenes strains in Brain Heart Infusion broth at 60 °C. Strains isolated from industrial environments MS22246 (●) and MS22248 (○), strains with pork origin MS22254 (▲) and MS22243 (△), strains with seafood origin MS22258 (■) and MS22261 (□). Level of detection is shown as a solid line at 1 log\(_{10}\) cfu/g.](image)

3.2 Moisture, salt and pH of applied foods

Before addition of salt, pork, chicken and salmon samples had average pH-values of 5.9 ± 0.02, 5.9 ± 0.11 and 6.3 ± 0.20, respectively. After addition of the quantity of salt listed in Table 1, pH-values decreased by 0.1 to 0.2 units.
The average moisture contents of pork, chicken and salmon samples were estimated from the declared nutritional composition to be 74, 75 and 68%, respectively (Table 1). Average WPS% concentrations were calculated based on these moisture contents and the quantities of salt added to 100 g food. The variation width of WPS% were approximated based on the known uncertainties stated in Table 1 to be as follows for: pork samples, 3.5 – 3.7% and 5.0 – 5.3%; and chicken samples 2.0 – 2.1%, 3.0 – 3.1%, 3.4 – 3.6% and 5.9 – 6.1%. For salmon samples, the standard deviation of the moisture content was unknown and the variation width was assumed to be similar to the values estimated for chicken.

### Table 2

Heat tolerance of *Listeria monocytogenes* expressed as D-values (min). D-values were calculated using the log-linear equation. Average values ± standard deviation of duplicate inactivation trials are given.

<table>
<thead>
<tr>
<th>Food</th>
<th>Strain(s)</th>
<th>WPS%</th>
<th>57 °C</th>
<th>58 °C</th>
<th>60 °C</th>
<th>61 °C</th>
<th>62.5 °C</th>
<th>65 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork</td>
<td>Cocktail</td>
<td>0</td>
<td>14 ± 1.0</td>
<td>-</td>
<td>5.3 ± 0.8</td>
<td>-</td>
<td>-</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6</td>
<td>31 ± 0.3</td>
<td>-</td>
<td>6.4 ± 0.8</td>
<td>-</td>
<td>-</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.2</td>
<td>46c</td>
<td>-</td>
<td>9.5c</td>
<td>-</td>
<td>-</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>MS22246</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>2.6d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6</td>
<td>-</td>
<td>-</td>
<td>3.9d</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Chicken</td>
<td>MS22246</td>
<td>0</td>
<td>-</td>
<td>19.8c</td>
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<td>4.4d</td>
<td>1.8c</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.9d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>-</td>
<td>33 ± 2.1</td>
<td>-</td>
<td>-</td>
<td>5.1c</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.2c</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>-</td>
<td>49c</td>
<td>-</td>
<td>-</td>
<td>7.8c</td>
<td>-</td>
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<tr>
<td></td>
<td>MS22258</td>
<td>0</td>
<td>-</td>
<td>7.7 ± 0.6</td>
<td>-</td>
<td>-</td>
<td>0.9 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>-</td>
<td>18 ± 1.3</td>
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<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>-</td>
<td>30 ± 6.4</td>
<td>-</td>
<td>-</td>
<td>4.6 ± 0.5 &amp; #400;</td>
<td>-</td>
</tr>
<tr>
<td>Salmon</td>
<td>MS22246</td>
<td>0</td>
<td>-</td>
<td>15 ± 0.9</td>
<td>4.3d</td>
<td>-</td>
<td>1.9 ± 0.1</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>-</td>
<td>40 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>6.1c</td>
<td>-</td>
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<td></td>
<td></td>
<td>6.0</td>
<td>-</td>
<td>44 ± 10</td>
<td>-</td>
<td>-</td>
<td>5.3 ± 0.3 &amp; #400;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MS22258</td>
<td>0</td>
<td>-</td>
<td>9.2 ± 1.1</td>
<td>-</td>
<td>-</td>
<td>1.2 ± 0.2</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>-</td>
<td>14 ± 2.6</td>
<td>-</td>
<td>-</td>
<td>1.8 ± 0.1</td>
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<td></td>
<td></td>
<td>6.0</td>
<td>-</td>
<td>31 ± 6.7</td>
<td>-</td>
<td>-</td>
<td>4.7 ± 0.7</td>
<td>-</td>
</tr>
</tbody>
</table>

- aMixture of strains MS22246 and MS22254
- bNo trials conducted
- cDuplicate trials were pooled as ≤ 3 log10-reduction was obtained in one of the trials
- dOne trial conducted
Figure 2. Survivor curves of a two-strain (MS22246 and MS22254) cocktail of *Listeria monocytogenes* in ground pork (○) and in ground pork with water phase salt of 3.6% (△) and 5.2% (□) exposed to heating at 57 °C (A), 60 °C (B) and 65 °C (C). Curves fitted with eq. 4, the Weibull model (solid lines) and curves fitted with eq. 2, the Log-linear model (dashed lines) are shown.
3.3 Heat tolerance of L. monocytogenes in foods

For the majority of the pork trials, the Geeraerd_shoulder model (eq. 3) or the Weibull model (eq. 4) fitted the data with root mean of squared errors (RMSE) that were lower than those observed for the log-linear model (eq. 2) (Table 3). AICc-values were applied to evaluate whether the log-linear model was unlikely compared to one of the non-linear models with a lower inactivation rate in the beginning than in the end of the heating process (Table 3). In three out of nine trials, the difference between AICc of the log-linear model and the Geeraerd_shoulder model or the Weibull model was more than 10 (Table 3), these trials being 57 °C and 60 °C without added salt and 60 °C with WPS of 3.6% (Figure 2). For these particular trials, the time to 1 log10-reduction cannot be expected to be accurately predicted with the log-linear model. However, since the objective was to develop a secondary model that is valid only for treatment scenarios where >3 log10-reduction is the goal, calculations showed that the log-linear model was adequate as the primary model in the present study. Based on this constraint, only survivor curves, where more than 3 log10-reduction were obtained within the time frame of the trial, were applied in the further model development. Data from trials, where less than a 3 log10-reduction was obtained in one of the duplicate trials, were pooled (Table 2) and used as a single trial for the further data analyses.

Heat tolerance of L. monocytogenes was expressed as D-values (eq. 6) estimated using the log-linear model. The D-values decreased with increasing temperatures and increased with increasing WPS% (Table 2). Variations for strains and foods were also found where the highest D-values were seen for strain MS22246 and the lowest when heating in pork (Table 2).

3.3 Salt-induced heat tolerance of L. monocytogenes in foods

The heat tolerance of L. monocytogenes increased with increasing levels of WPS% regardless of food, strain and heating temperature (Table 2). The salt-induced effect on the D-value was expressed as the relative increase (RI) i.e., estimating the factor by which the D-value will increase when salt is added to the food. A linear trend was observed when plotting RI-values against WPS% (Figure 3A). As the residuals appeared to increase with increasing WPS% (Figure 3B), the RI-values were square-root-transformed (Figures 3C & 3D) and ln-transformed (Figures 3E & 3F) to stabilize the variance. The ln-transformation resulted in the most randomly scattered residuals and was selected for the further analyses of results.

The effect of foods, strains and heating temperatures on the salt-induced heat tolerance, expressed as values of ln(RI), were evaluated by comparing CI95% of the slopes (1/WPS%) estimated by linear regression of sub-datasets. As shown in Figure 4, every CI95% overlapped with at least one other CI95%. For foods, the ln(RI) increased significantly faster with increasing WPS% in chicken compared to pork whereas CI95% for salmon, overlapped with CI95% for both chicken and pork. Strain MS22258 showed significantly higher
Table 3
Average ± standard error of parameter estimates for three primary inactivation models describing survival of *Listeria monocytogenes* heated at different temperatures in pork, having different water-phase-salt contents (WPS%).

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>WPS%</th>
<th>Inactivation model</th>
<th>No (log₁₀cfu/g)</th>
<th>SI (min)</th>
<th>kmax (1/min)</th>
<th>δ (min)</th>
<th>p</th>
<th>RMSEᵃ (log₁₀cfu/g)</th>
<th>AICc</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>0.2</td>
<td>Log-linear</td>
<td>7.77 ± 0.17</td>
<td>.ᵇ</td>
<td>0.17 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.44</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geeraerd_shoulder</td>
<td>7.28 ± 0.11</td>
<td>15.7 ± 2.5</td>
<td>0.22 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.25</td>
<td>8.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>7.33 ± 0.11</td>
<td>-</td>
<td>-</td>
<td>25.2 ± 2.4</td>
<td>1.70 ± 0.18</td>
<td>0.25</td>
<td>7.71</td>
</tr>
<tr>
<td>60</td>
<td>0.2</td>
<td>Log-linear</td>
<td>7.33 ± 0.10</td>
<td>-</td>
<td>0.05 ± 0.003</td>
<td>-</td>
<td>-</td>
<td>0.32</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geeraerd_shoulder</td>
<td>7.18 ± 0.12</td>
<td>27.5 ± 11</td>
<td>0.06 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.29</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>7.15 ± 0.12</td>
<td>-</td>
<td>-</td>
<td>61.3 ± 8.2</td>
<td>1.30 ± 0.17</td>
<td>0.30</td>
<td>20.1</td>
</tr>
<tr>
<td>65</td>
<td>0.2</td>
<td>Log-linear</td>
<td>7.44 ± 0.30</td>
<td>-</td>
<td>1.82 ± 0.18</td>
<td>-</td>
<td>-</td>
<td>0.56</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geeraerd_shoulder</td>
<td>7.10 ± 0.34</td>
<td>1.13 ± 0.7</td>
<td>2.13 ± 0.29</td>
<td>-</td>
<td>-</td>
<td>0.51</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>7.18 ± 0.35</td>
<td>-</td>
<td>-</td>
<td>1.89 ± 0.6</td>
<td>1.31 ± 0.29</td>
<td>0.53</td>
<td>24.0</td>
</tr>
<tr>
<td>3.6</td>
<td>0.2</td>
<td>Log-linear</td>
<td>7.47 ± 0.21</td>
<td>-</td>
<td>1.11 ± 0.08</td>
<td>-</td>
<td>-</td>
<td>0.40</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geeraerd_shoulder</td>
<td>7.21 ± 0.25</td>
<td>1.30 ± 0.8</td>
<td>1.23 ± 0.11</td>
<td>-</td>
<td>-</td>
<td>0.36</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>7.29 ± 0.26</td>
<td>-</td>
<td>-</td>
<td>2.66 ± 0.6</td>
<td>1.17 ± 0.17</td>
<td>0.39</td>
<td>17.3</td>
</tr>
<tr>
<td>5.2</td>
<td>0.2</td>
<td>Log-linear</td>
<td>7.36 ± 0.37</td>
<td>-</td>
<td>0.82 ± 0.10</td>
<td>-</td>
<td>-</td>
<td>0.76</td>
<td>29.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geeraerd_shoulder</td>
<td>7.16 ± 0.54</td>
<td>1.26 ± 2.3</td>
<td>0.87 ± 0.15</td>
<td>-</td>
<td>-</td>
<td>0.78</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>7.31 ± 0.52</td>
<td>-</td>
<td>-</td>
<td>3.05 ± 1.5</td>
<td>1.05 ± 0.31</td>
<td>0.80</td>
<td>34.6</td>
</tr>
</tbody>
</table>

ᵃRoot Mean Squared Errors as given in GinaFit
ᵇNot relevant for this model
Figure 3. Salt-induced heat tolerance of *Listeria monocytogenes* shown as the relative increase (RI) in D-values as a function of water phase salt % for pooled data from three foods, three strains and six heating temperatures. Different variance stabilizing transformations for RI and the corresponding residual plots are shown. A & B: no transformation, C & D: square root (\( \sqrt{\text{RI}} \)) transformation, E & F: natural logarithmic (ln) transformation. Adjusted R²-values, obtained from linear regressions (dotted lines), are stated.
effect on ln(\(RI\)) when compared to the mixture of strains MS22246 and MS22254 but overlapped with the
effect of strain MS22246 (Figure 4). Overall, no single effect was significantly different (\(P > 0.05\)) from the
others and all data were pooled for the further secondary modelling of the effect of WPS\% on the salt-
induced effect on heat tolerance of \(L.\ monocytogenes\).

**Figure 4.** Slope estimates derived from linear regression of the salt-induced heat tolerance of \(Listeria\)
\(monocytogenes\) (ln(\(RI\)) as a function of water-phase-salt \% for various sub-datasets. Bars represent the
95\% confidence intervals.

### 3.4 Secondary modelling and model evaluation

Fitting the effect of WPS\% on ln(\(RI\)) was based on the 41 observations with WPS\% ranging from 2.0 to 6.0.
The linear regression resulted in a standard error of the fit of 0.29, a significant slope (\(P < 0.001\)) with an
average of 0.208 and CI95\% from 0.141 to 0.275 and a non-significant intercept (\(P = 0.84\)) with an average
of -0.03 and CI95\% from -0.33 to 0.27. The final secondary model can be expressed as:

\[
\ln(\(RI\)) = 0.208 \times \text{WPS\%} - 0.03
\]

and is presented in Figure 5A. The performance of the model was evaluated using D-values found in the
scientific literature (Table 4). Four studies, where D-values had been determined in food-model systems
with and without added salt, were included. A total of 56 \(RI\)-values could be derived from the collected
data; 36 from crushed salmon roe (Li et al., 2017), 10 from minced beef (Jørgensen et al., 1995; Juneja et
al., 2013) and 10 from pork slurry (Lihoni et al., 2003). The graphical evaluation, comparing the
independent data to the model, showed that the literature derived data were inside, or very close to, the
CI95\% bands of the model except for one \(RI\)-value for salmon roe containing 4.2 WPS\%, which was located
so far from the other data that it was suspected to be an outlier (Figure 5B). The evaluation resulted in \(B_f\)
### Table 4

Overview of the data included in the evaluation of the developed secondary model for prediction of the salt-induced effect on the heat tolerance of *Listeria monocytogenes*.

<table>
<thead>
<tr>
<th>Food</th>
<th>Stain(s)</th>
<th>Added salt* (%)</th>
<th>Assumed moisture (%)</th>
<th>WPS (%)</th>
<th>D-value (min)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon roe</td>
<td>F2365</td>
<td>0.17e</td>
<td>76e</td>
<td>0.2</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.67</td>
<td>75</td>
<td>2.2</td>
<td>5.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.17</td>
<td>73</td>
<td>4.2</td>
<td>9.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.67</td>
<td>72</td>
<td>6.1</td>
<td>12</td>
<td>5.6</td>
</tr>
<tr>
<td>F4260</td>
<td>0.17c</td>
<td>76c</td>
<td>0.2</td>
<td>6.7</td>
<td>1.4</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.67</td>
<td>75</td>
<td>2.2</td>
<td>11</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.17</td>
<td>73</td>
<td>4.2</td>
<td>17</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.67</td>
<td>72</td>
<td>6.1</td>
<td>20</td>
<td>6.8</td>
</tr>
<tr>
<td>V7</td>
<td>0.17c</td>
<td>76c</td>
<td>0.2</td>
<td>4.0</td>
<td>1.4</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.67</td>
<td>75</td>
<td>2.2</td>
<td>9.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.17</td>
<td>73</td>
<td>4.2</td>
<td>11</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.67</td>
<td>72</td>
<td>6.1</td>
<td>17</td>
<td>5.0</td>
</tr>
<tr>
<td>Minced beef</td>
<td>Cocktaild</td>
<td>0.17e</td>
<td>58e</td>
<td>0.3</td>
<td>9.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.17</td>
<td>58</td>
<td>2.0</td>
<td>10</td>
<td>4.7</td>
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<tr>
<td></td>
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<td>2.17</td>
<td>58</td>
<td>3.6</td>
<td>19</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.17</td>
<td>58</td>
<td>5.2</td>
<td>21</td>
<td>9.3</td>
</tr>
<tr>
<td>Scott A</td>
<td>0.15g</td>
<td>71g</td>
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<td>-</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>9.0</td>
<td>-</td>
<td>3.9</td>
<td>-</td>
</tr>
<tr>
<td>Pork slurry</td>
<td>ATCC 19116 (starved)</td>
<td>0.02h</td>
<td>95h</td>
<td>0.02</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (stationary)</td>
<td>0.02h</td>
<td>95h</td>
<td>0.02</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (exponential)</td>
<td>0.02h</td>
<td>95h</td>
<td>0.02</td>
<td>-</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (starved)</td>
<td>3.02</td>
<td>92</td>
<td>3.2</td>
<td>7.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (stationary)</td>
<td>3.02</td>
<td>92</td>
<td>3.2</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (exponential)</td>
<td>3.02</td>
<td>92</td>
<td>3.2</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (starved)</td>
<td>6.02</td>
<td>89</td>
<td>6.3</td>
<td>15</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (stationary)</td>
<td>6.02</td>
<td>89</td>
<td>6.3</td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>ATCC 19116 (exponential)</td>
<td>6.02</td>
<td>89</td>
<td>6.3</td>
<td>-</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a* Denotes the total salt content from the endogenous and added salt in the products  
*c* Based on values from https://fdc.nal.usda.gov/fdc-app.html#/food-details/175132/nutrients  
*d* Scott A, MF27137, MF38521 and MF46869  
*e* Based on values from https://fdc.nal.usda.gov/fdc-app.html#/food-details/174037/nutrients  
*f* No value available  
*g* Based on values from https://frida.fooddata.dk/food/891  
*h* Based on values from https://fdc.nal.usda.gov/fdc-app.html#/food-details/167902/nutrients

and $A_r$ of 0.87 and 1.28, respectively, which were slightly improved to 0.89 and 1.26, respectively, when excluding the suspected outlier (Table 5). The proportion of data outside the CI95% bands, was identical for
the dataset used for model development and the dataset used for model validation (Table 5). Over-
predicted values, were mainly from beef studies which resulted in identical $B_f$ and $A_f$ of 1.24 (Table 5).
Under-predicted values were partly from salmon roe and partly from pork slurry (Figure 5B). This
observation was reflected in $B_f$-values below 1.0 for data from both salmon roe as well as pork slurry
studies i.e., 0.82 (Table 5).

Figure 5. Graphical illustration of the developed secondary model for prediction of the salt-induced heat
tolerance ($\ln(\text{RI})$) of *Listeria monocytogenes* in meat and seafood products as a function of water-phase-salt
% (A) and the model evaluation with literature data (B). The solid line is the fitted model and the dotted
lines represent the lower and upper 95 % confidence bands. The • symbols are observed data, ○ are data
from crushed salmon roe (Li et al. 2017), □ are data from minced beef (Juneja et al. 2013; Jørgensen et al.
1995) and △ are data from pork slurry (Lihono et al. 2003).

Table 5
Validation of the developed secondary model for prediction of the salt-induced effect on heat
tolerance of *Listeria monocytogenes* using bias ($B_f$) and accuracy factors ($A_f$).

<table>
<thead>
<tr>
<th>Data</th>
<th>Number of samples</th>
<th>$B_f$</th>
<th>$A_f$</th>
<th>% samples inside CI95%$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All literature data</td>
<td>56</td>
<td>0.87</td>
<td>1.28</td>
<td>96</td>
</tr>
<tr>
<td>Without outlier$^a$</td>
<td>55</td>
<td>0.89</td>
<td>1.26</td>
<td>98</td>
</tr>
<tr>
<td>Salmon roe (crushed)$^a$</td>
<td>35</td>
<td>0.82</td>
<td>1.24</td>
<td>97</td>
</tr>
<tr>
<td>Beef (minced)</td>
<td>10</td>
<td>1.24</td>
<td>1.24</td>
<td>100</td>
</tr>
<tr>
<td>Pork (slurry)</td>
<td>10</td>
<td>0.82</td>
<td>1.37</td>
<td>100</td>
</tr>
<tr>
<td>Data used for modelling</td>
<td>41</td>
<td>1.00</td>
<td>1.26</td>
<td>95</td>
</tr>
</tbody>
</table>

$^a$The outlier marked in Figure 5B has been excluded
$^b$95% confidence interval
4. Discussion

4.1 Selection of suitable strains for heat inactivation trials

The two strains isolated from industrial environments (MS22246 and MS22248) showed the highest $D_{60}$-values in BHI broth being approximately two- to seven-fold higher than $D_{60}$-values of the meat isolates (MS22243 and MS22254) and seafood isolates (MS22258 and MS22261) (Figure 1). This suggested that routine exposure of the multiple environmental stresses that occur during food processing, such as heat, acid, osmotic and pressure stresses, as well as cleaning detergents and sanitizers for the processing equipment, might result in enhanced heat tolerance of $L. monocytogenes$ (Thévenot et al., 2006).

Li et al. (2017) suggested that the most heat tolerant $L. monocytogenes$ strain should be the target for designing thermal processes for foods. Assuming that this also applies when developing predictive models, the strains with the highest $D_{60}$-values in BHI broth from each origin were selected for the heat inactivation trials in foods in the present study (Figure 1). However, the present study focused on quantifying the effect of increasing concentrations of salt on the heat tolerance of $L. monocytogenes$. This involves the ability of a strain to respond to the combination of heat and osmotic stress, which cannot necessarily be derived from behaviour in foods without added salt. Hence, it may not be the most heat tolerant strain identified in an unsalted heating menstruum that also has the best ability to respond to the combination of heat and added salt. Therefore, three strains, as opposed to one, were applied for the model development in the study. Figure 4, which depicts the observed variability in salt-induced change in heat tolerance, illustrates the importance of this decision. Notably, the strain isolated from seafood (MS22258), which had lower heat tolerance than the environmental (MS22246) strain when heated in unsalted foods (Table 2), tended to increase its heat tolerance more, i.e., larger increase in $RI$, with increasing WPS% compared to the environmental MS22246 strain (Figure 4). This result underpins that more than one strain should be included in studies to quantify the effects of other stresses on heat tolerance of bacteria as also recommended in a recent review (den Besten et al., 2018).

4.2 Estimating heat tolerance of $L. monocytogenes$

The traditional approach to describe microbial decay as first-order kinetics is based on the assumption that all bacterial cells within a population are identical and have the same heat tolerance. However, as indicated by the observed AICc (Table 3), inactivation models having a “shoulder” i.e., a slower inactivation in the beginning of the heat treatment, fitted some of the survivor curves better than the log-linear model (Figure 2). These characteristics are well-known for survivor curves of $L. monocytogenes$ (Cole et al., 1993; Jørgensen et al., 1995; Juneja and Eblen, 1999). The presence of sub-populations with different sensitivities to heat and osmotic stresses and ensuing differences in their initial survival, was especially observed in
trials carried out at lower heating temperatures and lower WPS% (Figure 2). Under practical conditions
these results imply that using log-linear D-values for designing safe heating processes may give fail-
dangerous results, in particular, if the goal is to achieve a 1 to 3 log₁₀-reduction. However, the objective of
the model developed in the present study was to ensure the safety of heat-treated meat and seafood
products with extended refrigerated shelf-life. As it has been shown that heat treatments resulting in 3 – 4
log₁₀-reduction of *L. monocytogenes* prevented its regrowth during subsequent chilled storage for up to 30
days (Hansen and Knøchel, 2001), it was decided that applicability of the secondary model to predict the
salt-induced change in heat tolerance should be limited to treatments resulting in at least a 3 log₁₀-
reduction. Therefore, solely survivor curves, where more than 3 log₁₀-reduction were obtained, were
applied for exploring and quantifying the effect of salt on heat tolerance of *L. monocytogenes* in pork,
chicken and salmon. This constraint was applied in order to use the same log-linear approach for estimating
D-values for all trials.

4.3 Effect of salt on heat tolerance

As also reported by Doyle et al. (2001), salt protected against heat inactivation of all three strains of *L.
*monocytogenes*, in all three types of food and at all temperatures, meaning that increasing D-values were
found with increasing WPS% (Table 2). Relative increases of D-values, resulting from the addition of salt,
were up to six-fold depending on the exact WPS% (Figure 5A). A clear salt-induced effect was observed for
WPS above 2% (Figure 5A). These findings were in agreement with previous studies conducted in foods
(Farber et al., 1989, Juneja et al., 2013; Li et al., 2017; Lihono et al., 2003; Yen et al., 1991). Salt-induced
increased in D-values ranged from two- to four-fold in minced beef (Jørgensen et al., 1995; Juneja et al.,
2013), and two- to six-fold in crushed salmon roe (Li et al., 2017) and pork slurry (Lihono et al., 2003)
(Figure 5B). Another study reported even higher increases of up to eight-fold in ground meat after the
addition of a curing mixture (Farber et al., 1989), indicating that other ingredients than NaCl in the curing
mixture influenced the heat resistance of *L. monocytogenes*. In fact, Yen et al. (1991) demonstrated that a
combination of NaCl, dextrose, phosphates, erythorbate and NaNO₂ increased the heat tolerance of *L.
monocytogenes* in ground pork more than any one of the ingredients alone. The increased heat tolerance
of *L. monocytogenes* may be explained by the reduction of water activity in the heating menstruum
(Mackey et al., 1990). Also, an increase in WPS% will elicit the osmotic stress response in *L. monocytogenes*
causing it to accumulate osmolytes such as glycine betaine and carnitine, which are present in plant and
meat-based foods, respectively (Angelidis and Smith, 2003; Meadows and Wargo, 2015). Salt exposure and
osmolytes accumulation will lead to cross-protection against other stresses including heat (Lou and Yousef,
1997) and low temperatures (Bayles and Wilkinson, 2000; Smith, 1996; Yancey et al., 1982) and, therefore,
may play a part in the observed salt-induced heat tolerance of *L. monocytogenes*. 

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4.4 Predicting the effect of salt on heat tolerance

Secondary predictive models describing the combined effect of salt and heating temperature on heat tolerance of *L. monocytogenes* have previously been published for broth (Cole et al., 1993), beef gravy (Juneja and Eblen, 1999), pork slurry (Lihono et al., 2003), minced beef (Juneja et al., 2013) and salmon roe (Li et al., 2017). Common for all of them are that polynomial equations were applied for modelling the rate of inactivation of *L. monocytogenes*. In the present study, it was hypothesized that the rise in the heat tolerance, gained when salt is present during heat exposure, is independent of heating temperature, strain and food. Therefore, the relative increase in heat tolerance (RI) obtained when salt had been added to the food was modelled as a function of WPS%. Large variation was observed, which also tended to increase with increasing WPS% (Figure 3A). The ln-transformation was the best choice for stabilizing the variance (Figures 3E & 3F). Using the ln-transformation, it was tested whether part of the variance could be explained by type of food, strain or heating temperature. No single factor was statistically significantly different from the others, although, the rise in RI with increasing levels of WPS% tended to be lower in pork (Figure 4) indicating that one or more non-controlled intrinsic factors might have influenced the results and require moderation of the hypothesis. Further product characterization may help to reveal if other intrinsic factors have an effect on the heat tolerance of *L. monocytogenes* and, therefore, should be included in future improvements of the model. An obvious intrinsic factor could be pH. It is well-described that pH of foods affects the heat tolerance of *L. monocytogenes* and that lowering of pH will result in faster inactivation (Cole et al., 1993, Juneja and Eblen, 1999). It is, however, not so clear how lowering of pH affects the salt-induced heat tolerance of *L. monocytogenes*. Two of the previously published studies (Cole et al., 1993; Lihono et al., 2003) have included the effect of pH in the polynomial model but both studies used second order equations, i.e., the triple interaction term of salt x temperature x pH was not estimated.

The web-based ComBase platform includes a broth model for prediction of thermal inactivation of *L. monocytogenes*. Using this model, and assessing the pH-values of the foods applied in the present study, the differences appeared to be too small to expect any differences in the salt-induced heat tolerance (results not shown). Thus, it is unlikely that the observed effect was a result of pH-differences. Other constituents in meat and seafood that have been shown to affect heat tolerance of *L. monocytogenes* are fat content (Ben Embarek and Huss, 1993; Fain et al., 1991) and lactic acid content (Jørgensen et al., 1999). However, no studies investigating the combined effect of any of these factors with the salt and heating temperature have been identified making it difficult to pursue this further.

As the data for foods without added salt were used as denominator when estimating the response variable used for secondary modelling, these were excluded from the modelling leaving a pool of 41 observations with WPS% from 2 to 6 for the fitting process (Figure 5A). On account of the large variation, it was chosen...
to include the 95% confidence bands as part of the model evaluation. The validation dataset, comprising heat tolerance data from three foods, eight strains and four heating temperatures (Table 4), displayed variation to the same extent as the observed data (Figure 5B). This suggested that the newly developed model covered sufficiently the biological variation to be considered a generic model. The $B_f$-value of the model evaluation was 0.89 (Table 5), which was lower than 1, indicating that the model was slightly fail-dangerous as it under-predicted the relative increase of heat tolerance of *L. monocytogenes* when exposed to salt. Performance at this level would be perceived as acceptable for models predicting lag time durations or growth rates (Ross, 1996). Also, the $A_f$-value of 1.26 was within the acceptable range for growth parameters (Ross, 1996) showing that the predicted values on average were 26% wrong. Evaluating the sub-datasets, revealed that $R_I$ was under-predicted to an unacceptable extent for pork slurry and crushed salmon roe as $B_f = 0.82$ (Table 5) was lower than 0.87 (Ross, 1996). Since the $B_f$ and $A_f$ indices originally were introduced for validating prediction of growth parameters and not parameters related to inactivation, the acceptability limits may, however, not be applicable. For prediction of heat inactivation rates there has been a tradition of being conservative using worst case scenarios i.e., designing thermal processes based on the highest observed D-value or the upper 95% confidence band from a meta-analysis (Van Asselt and Zwietering, 2006). Alternatively, the dependent and independent data could be pooled to find new and more robust parameter estimates covering even more biological variation.

In conclusion, a secondary model was developed that describes the salt-induced increase in heat tolerance (expressed as D-values) for *L. monocytogenes* being processed in meat and seafood products with WPS% ranging from 0.2 to 6%. This model can be used to guide the design of safe heat processes, with the objective of achieving > 3 log$_{10}$ reductions, for manufacturers of lightly preserved and mildly processed products with extended refrigerated shelf-life. The model was developed based on the hypothesis that the factor, by which the D-value of *L. monocytogenes* increases when salt is added to a food, solely depends on product WPS% thereby being independent of strain, heating temperature and food. Although, the secondary model captured a significant portion of the salt-induced change in D-values for the tested products with bias and accuracy factors of 0.89 and 1.26, respectively, it is recommended to apply the upper 95% confidence band of the model to ensure that all biological variance is captured. Future studies could reveal if there are other intrinsic food factors, which influence heat tolerance of *L. monocytogenes* and could be used to for further improvement of the model.

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Data statement

The data will be made available upon request.

Declaration of competing interest

None.

References


