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Enhanced heat resistance of *Listeria innocua* as a surrogate of *Listeria monocytogenes* after sublethal heat treatment

O. Haykir[1*] , Cs. Mohácsi-Farkas[1] and T. Engelhardt[2]

[1] Department of Food Microbiology, Hygiene and Safety, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences (MATE), Somlói út 14–16, 1118, Budapest, Hungary

[2] Digital Food Chain Education, Research, Development and Innovation Institute, University of Veterinary Medicine, István u. 2, 1078, Budapest, Hungary

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ABSTRACT

Its ability to survive under different environmental conditions makes *Listeria monocytogenes* a critical concern for food safety. When the microorganisms are exposed to sublethal heat treatment above their optimum growth temperature, they increase stress adaptation for further heat treatments. In order to investigate heat stress resistance of *L. monocytogenes*, *L. innocua* as a surrogate was exposed to sublethal heat at 46 °C for 30 and 60 min, prior to heat treatment at 60 °C. There was no significant difference in $D_{60°C}$ values between samples exposed to sublethal heat for 30 min and non-pre-heat-treated samples (control) ($P > 0.05$). In comparison, sublethal heat treatment for 60 min caused a significant increase in $D_{60°C}$ values compared to control samples ($P < 0.05$). Additionally, cluster analysis of mass spectra obtained from MALDI-TOF was analysed by discriminant analysis of principal components (DAPC) for sublethal heat treatment at 46 °C for 30 min and control group to check stress response at the proteomic level. However, differentiation of stress responses by distinct clusters was not revealing.

KEYWORDS

* Corresponding author. Tel.: +36-70-742-9743. E-mail: oktay.haykir@gmail.com

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1. INTRODUCTION

Because of its high fatality rate, survival in different environmental conditions such as refrigeration temperatures, acidic foods, high salt content, *Listeria monocytogenes* is a considerable concern in the food industry (Doyle et al., 2001; Gandhi and Chikindas, 2007; Sergelidis and Abrahim, 2009). In 2018, a multi-country outbreak of *Listeria monocytogenes* serogroup IVb, caused by frozen vegetables produced in Hungary, was reported. According to the report, the strain remained persistent after cleaning and sanitisation treatment in the factory for years. This outbreak caused 47 cases with nine deaths (case fatality rate 19%) (European Food Safety Authority (EFSA), 2018).

Yousef and Courtney (2002) define stress as any deleterious factor or condition that negatively influences microbial growth or survival. Microorganisms respond to these stresses in various ways, such as protein synthesis for damage and cell transformation to a viable but non-culturable state (VBNC). These responses enhance the tolerance of the microorganisms to the following same or different types of stress. This phenomenon is called stress adaptation (Sergelidis and Abrahim, 2009). Stress adaptation to heat and other types of stresses, its mechanisms and its impact on the food industry have been reviewed previously (Doyle et al., 2001; Gandhi and Chikindas, 2007; NicAogáin and O'Byrne, 2016; Bucur et al., 2018). Strain variation, age of microorganism, growth conditions, test conditions, and food matrix are the main factors that affect heat resistance of *L. monocytogenes* and other microorganisms (Doyle et al., 2001).

Physiological cellular responses like thermotolerance are caused by the synthesis of different proteins called heat shock proteins (HSPs). These heat-shock proteins are mainly protein chaperons that cause the folding and assembly of damaged proteins. Stress adaptation causes changes in protein expression, which are reflected in the proteome of the microorganisms. For this reason, investigating the proteome of the microorganisms under various stress conditions can give illustrative thoughts about stress adaptation of the microorganisms. Since it is much faster than two-dimensional gel electrophoresis (2D GE) and able to catch low molecular weight stress proteins, Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS) protein profiling also makes it possible to detect stress responses. Ribosomal and cell structure proteins show up as peaks in MALDI-TOF mass spectra. The changes in the characteristics of the peaks enable us to analyse the stress response of the microorganisms (Schott et al., 2016).

The overall objective of this study was to investigate the enhanced heat tolerance of *L. innocua* as a surrogate of *L. monocytogenes* after sub-lethal heat exposure. The aim of the current work focused on the survival characteristics and changes in the proteome of the bacterium.

2. MATERIALS AND METHODS

2.1. Strain

Listeria innocua strain (T1), a strain from the collection of MATE, was inoculated into Trypto-Casein Soy Broth (TSB, Biokar, France) (pH 7.3) at 37 °C to yield a cell population of approximately 8 lg CFU mL^{-1}.

2.2. Identification of enhanced heat resistance

Sublethal stress conditions were applied according to the protocol of Ágoston et al. (2010). The time-temperature combinations used for sub-lethal heat stress were 46 °C for 30 and 60 min. After exposure to the sublethal heat stress, the samples were immediately transferred to a water bath (Haake, Germany) set appropriately so that the samples were exposed to temperatures of 60 °C for 0, 3, 6, and 9 min. For control, samples without prior heat treatment were placed into the water bath at 60 °C. The heat-treated samples were immediately placed into an ice-bath prior to serial dilution in tubes containing 9 mL of peptone-NaCl (0.85%) buffer. The diluted samples were spread plated on Trypto-Casein Soy Agar (TSA, Biokar, France). The plates were incubated for 24 h at 37 °C prior to enumeration.

2.3. Sample preparation and MALDI-TOF MS analysis

Sample preparation for cluster analysis of MALDI-TOF peaks was made with modifications to the previous work of Schott et al. (2016). In order to obtain mass spectra, samples were taken from a water bath every 3 min for 15 min for both control and sub-lethal heat exposed samples for 46 °C for 30 min. Mass spectra of the samples for mass range 2–20 kDa are obtained in MALDI-TOF MS (BrukerDaltonics, Bremen, Germany) by 280 accumulated laser shots. All experiments were carried out in triplicates on three different days.

2.4. Data processing and statistical analysis

2.4.1. Determination of D-value. The $D_{60°C}$ -values of the strain after the sub-lethal heat exposure of 30 and 60 min at 46 °C with the control samples were calculated to determine whether the sub-lethal heat exposure increased the D-value as previously described by Farber and Brown (1990).

2.4.2. Cluster analysis of the peaks from MALDI-TOF MS. The baseline of the exported mass spectra of each sample was subtracted as pre-processing. Peak-based cluster analysis was applied to these pre-processed mass spectra to illustrate the stress response dynamics. Visualisation by dendrogram was carried out with the KNIME Analytics Platform (Version 4.2.1) with R Foundation for Statistical Computing (Vienna, Austria). A package for RStudio software, adegenet was used for discriminant analysis of principal components (DAPC) to analyse the differentiation of stress responses (Jombart, 2008). Clusters were defined as previously described by Schott et al. (2016). In the end, DAPC maintains a barplot of eigenvalues and a scatterplot representing individuals as dots and groups as inertia ellipses.

3. RESULTS AND DISCUSSION

The inactivation kinetics of *L. innocua* T1 after the sub-lethal heat exposure for 30 and 60 min and without prior exposure (control) are shown in Figs 1 and 2, respectively.

Table 1 shows $D_{60°C}$ values of sub-lethal heat exposed samples at 46 °C for 30 and 60 min and non-prior heat exposured (control) samples. There was no significant difference in $D_{60°C}$ values between the samples after prior exposure of 46 °C for 30 min and the control ($P > 0.05$). Instead, treatment of 46 °C for 60 min enhanced survival of the strain at 60 °C ($P < 0.05$).
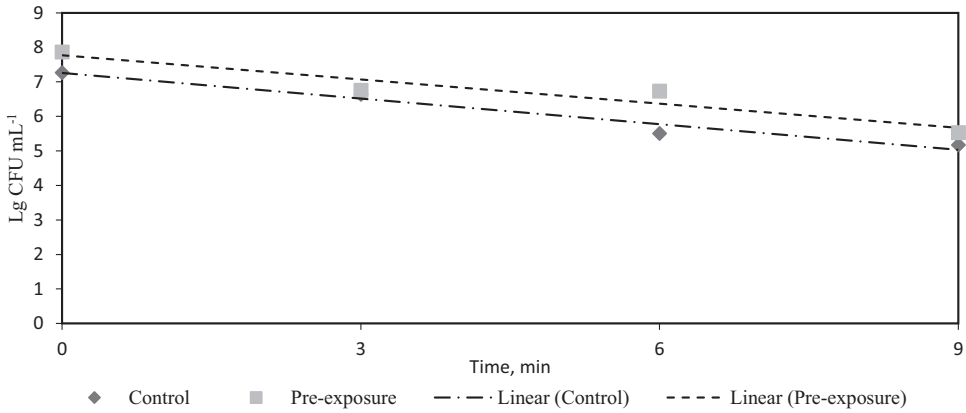
Fig. 1. Enhanced heat resistance of *L. innocua* T1 at 60 °C when pre-exposed for 30 min at 46 °C, $y_{\text{control}} = 0.2479x + 7.2605$, $R^2_{\text{control}} = 0.9628$, $D_{60°C \text{ control}} = 4.03$ min, $y_{\text{pre-exposure at 46°C for 30 min}} = 0.2345x + 7.774$, $R^2_{\text{pre-exposure at 46°C for 30 min}} = 0.9043$, $D_{60°C, \text{ pre-exposure at 46°C for 30 min}} = 4.26$ min
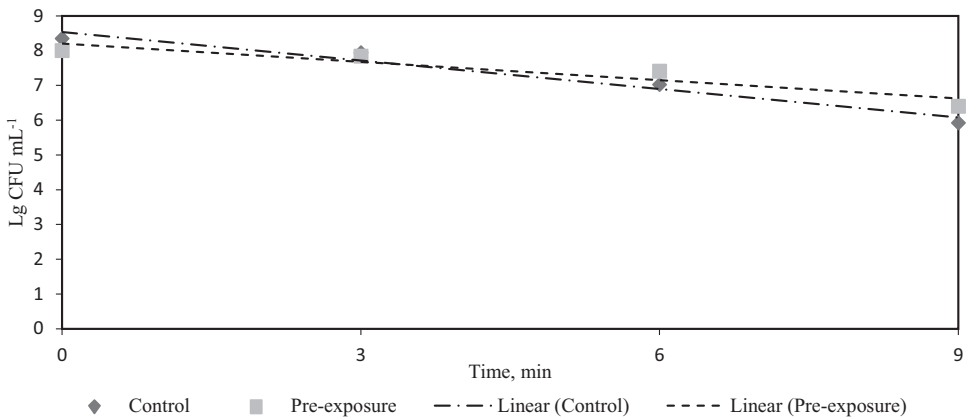

Fig. 2. Enhanced heat resistance of *L. innocua* T1 at 60 °C when pre-exposed for 60 min at 46 °C, $y_{\text{control}} = 0.2732x + 8.5395$, $R^2_{\text{control}} = 0.9639$, $D_{60°C, \text{ control}} = 3.66$ min, $y_{\text{pre-exposure at 46°C for 60 min}} = 0.1751x + 8.2021$, $R^2_{\text{pre-exposure at 46°C for 60 min}} = 0.8833$, $D_{60°C, \text{ pre-exposure at 46°C for 60 min}} = 5.71$ min

Table 1. Effect of pre-exposure to sub-lethal temperatures of 46 °C for 30 and 60 min on the $D_{60°C}$ values for *Listeria innocua* T1 in Tryptic Soy Broth

Exposure time to sub-lethal temperature (min)	$D_{60°C}$ values (±Standard Deviation) (min)	
	Control	Pre-exposed
30	4.03 (±1.06)[a]	4.26 (±0.36)[a]
60	3.66 (±0.47)[a]	5.71 (±0.85)[b]

[a-b] For each row, different superscripts denote statistically significant difference $P < 0.05$.

Thirty-four mass spectra from control samples and sub-lethal heat exposed samples at 46 °C for 30 min were analysed to check possible differentiation at the proteome level. Of these 34 samples, 18 belong to sub-lethal heat treatment samples, while 16 of these samples belong to control samples. Two samples from the control group were excluded since no mass spectra were obtained because of a mistake during the sample preparation procedure. These thirty-four samples were grouped into three different clusters (Fig. 3). This separation lies in the mathe-matical approach, discriminant analysis of principal components (DAPC). Briefly, Principal Component Analysis (PCA) is a statistical method that looks at each spectrum with the most variation, while Discriminant Analysis (DA) aims to maximise the separation of known cate-gories. If we look at these clusters, cluster 1 contains 12 samples, cluster 2 contains four samples, and cluster 3 contains 18 samples. Seven of the control samples were in the first cluster, two were in the second cluster, and seven were in the third cluster. For the sub-lethal heat-treated samples, five were in the first cluster, two were in the second cluster, and eleven were in the third cluster (data not shown). Overall, there was no meaningful separation of the samples into the different groups.

Ágoston et al. (2010) studied enhanced heat resistance on *L. monocytogenes* after prior heat exposure to 46 °C for 30 and 60 min. There were 1.7 and 5.3 fold increase in $D_{60°C}$ values, respectively, compared to the samples heated at 60 °C without prior heat-treatment. However, there was a decrease in heat resistance after the treatment of 50 °C for 60 min, compared to 30 min. They concluded an upper limit in terms of sub-lethal heat resistance. Farber and Brown (1990) investigated the effect of prior exposure of different *L. monocytogenes* strains to sub-lethal
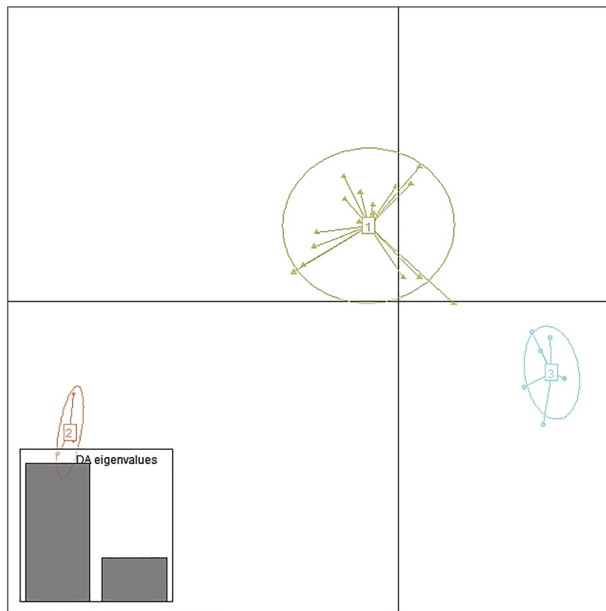

Fig. 3. Clusters of the MALDI-TOF MS peaks, obtained from the DAPC analysis of the peaks from control and pre-heat exposed samples at 46 °C to 30 min

temperature exposure in sausage mix. No significant difference was reported in $D_{64°C}$ values when they treated samples at 48 °C for 30 and 60 min. Instead, there was a 2.4 fold increase in $D_{64°C}$ values after the pre-exposure of 48 °C for 120 min, compared to untreated cells. In our study, there was no significant increment in $D_{60°C}$ values after sub-lethal heat treatment at 46 °C for 30 min, while there was a 1.6 fold increase in $D_{60°C}$ values after 60 min of treatment. Shen et al. (2014) examined $D_{60°C}$ values of 3 different heat-tolerant strains of *L. monocytogenes* to search for enhanced heat adaptation after subjecting to sublethal heat stress at 46 °C for 30 and 60 min. There was a significant heat resistance in all strains after 30 and 60 min sublethal heat treatment. However, sublethal heat treatment for 90 min caused no significant difference in $D_{60°C}$ value in one of the strains, compared to control. Also no difference in $D_{60°C}$ value after 15 min of heat treatment was found in one of the strains. Our results and previous results show that heat stress adaptation depends on the duration of sublethal heat exposure. Stephens et al. (1994) demonstrated the food safety risk in slow-cooked foods by investigating the effect of heating rate on the thermal inactivation of *L. monocytogenes*.

Sörqvist (2003) stated that to use an indicator organism to evaluate the heat resistance of an actual microorganism, indicator bacteria should have equal or more heat resistance. It is suggested that the heat resistance of *L. innocua* may have higher average heat resistance than *L. monocytogenes*. It is also stated that research on the heat resistance of *L. innocua* as a surrogate is insufficient. Therefore, non-pathogenic *L. innocua* is used in our research as an indicator for heat resistance of *L. monocytogenes*.

As said before, there are some genomic and proteomic responses after exposing *L. monocytogenes* to sublethal heat temperature conditions. Van der Veen et al. (2007) showed that some genes of *L. monocytogenes* were differentially expressed when the cells were exposed to a prior sublethal temperature of 48 °C. In similar research done by Agoston et al. (2009), 20 proteins were differentially expressed (ten up-regulated and ten down-regulated) when cells were initially exposed to 48 °C for 30 min before 60 °C for 9 min. In order to investigate the changes on the molecular level in *L. innocua* after sublethal heat exposure of 46 °C for 30 min, mass spectra obtained from MALDI-TOF were analysed in our research. The hypothesis was that after the changes in the proteome of the samples, control and pre-heat exposed samples might be positioned in different clusters. Alternatively, that samples could be separated from each other after a particular exposure time, so it can be proven that there is a specific time needed for the changes in the proteome of the samples to appear. However, the separation of the samples did not give a revealing result. The three clusters were not meaningful, proving our results that there was no significant difference ($P > 0.05$) in the $D_{60°C}$ values of sublethal heat-treated samples for 46 °C for 30 min and control samples. According to our knowledge, this is the first research on peak based cluster analysis of heat stressed samples of *L. innocua* or *L. monocytogenes*.

4. CONCLUSIONS

Enhanced heat resistance of *L. innocua* T1 as a surrogate of *L. monocytogenes* was investigated. There was no significant difference in $D_{60°C}$ values between control samples and sublethal heat exposed samples at 46 °C for 30 min ($P > 0.05$). Instead, when the cells were exposed to 46 °C for 60 min, there was a significant increase in the $D_{60°C}$ value ($P < 0.05$). Additionally, no meaningful differentiation was found after analysing MALDI-TOF MS spectra of control and

sublethal heat exposed samples at 46 °C for 30 min. These results suggest that heat stress adaptation depends on the duration of the heat treatment.

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