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ABSTRACT

The aim of this study was to evaluate the effect of lactic acid (LA) with and without organic material at various post-treatment recovery times on the heat resistance of *Listeria monocytogenes* (Lm). LA decreased Lm numbers; however, the effect was remarkably attenuated by the presence of organic matter. Five strains of Lm were treated with LA and the listericidal effects were compared. The effect of LA varied depending on the strain, with ≥ 3.0% (w/w) LA required to kill the Lm strains in a short time. The heat resistance of Lm treated with LA was examined with respect to the time interval between the acid treatment and the subsequent manufacturing step. The heat resistance of Lm was shown to significantly increase during the post-treatment period. Heat tolerance (D value) increased up to 3.4-fold compared with the non-treated control bacteria. RNA sequencing and RT-PCR analyses suggested that several stress chaperones, proteins controlled by RecA and associated with high-temperature survival, were involved in the mechanism of enhanced heat resistance. These results are applicable to manufacturers when LA and heat treatment methods are utilized for the effective control of Lm in foods.

1. Introduction

Listeria monocytogenes (Lm) has been recognized as a foodborne pathogen since the early 1980s and many outbreaks of listeriosis and sporadic cases continue to occur (Barkley et al., 2016; Laksanalamai et al., 2012; Lassen et al., 2016; Stephan et al., 2015; Thomas et al., 2015). Lm is a rod-shaped, Gram-positive, facultatively anaerobic bacterium that is ubiquitously found in the environment, including at food manufacturing sites. Lm is capable of growing at refrigeration temperatures, high salt concentrations and a wide pH range (Farber and Peterkin, 1991; Ferreira et al., 2014). These characteristics enable the contamination and growth of this pathogen in food and food manufacturing sites and are factors in the challenge of controlling Lm. Many reports have investigated the control of Lm in foods and/or media based on hurdle technology (Ahamad and Marth, 1989; Buchanan et al., 1993, 1994; Carlier et al., 1996; Faleiro et al., 2003; Francis and O'Beirne, 2005; Foegeding and Leasor, 1990; Lunden et al., 2008; Schirmer et al., 2014).

Organic acids, especially lactic acid, are used to sanitize livestock carcasses (Official Journal of the European Union, 2013; USDA-FSIS, 2016) and are added to foods to prevent the growth of Lm (Byelashov et al., 2010; Wemmenhove et al., 2016; Yoon et al., 2011). However, there are no reports showing systematically the bactericidal effect of LA at different concentrations using multiple strains of Lm. There are various reports on the risk of Lm after acid treatment. Skandamis et al. (2008) reported that Lm treated at pH 5.0 (adjusted with HCl) for 1 or 1.5 h exhibited increased resistance to acid treatment at pH 3.5 (adjusted with lactic acid) or heating at 57 °C. Similar results have been reported elsewhere (Davis et al., 1996; O'Driscoll et al., 1996; Phan-Thanh et al., 2000). Although these effects occurred when Lm was treated with acid for about 1 to 2 h, the effect of short-term treatment, i.e., several seconds to several minutes, has not been reported.

Ferreira et al. (2001) suggested that improved acid resistance was due to SigB, which is a major regulator of the stress response. Genes regulated by SigB have been reported to be involved in various stress responses (Abram et al., 2008; Ait-Ouazzou et al., 2012; Palmer et al., 2009; Raimann et al., 2009; Utratna et al., 2011; Zhang et al., 2011). Furthermore, RNA sequencing (RNA-seq) has been employed to assess responses to other stresses such as disinfectants, growth conditions and so on (Casey et al., 2014; Lobel and Herskovits, 2016; Oliver et al.,

Abbreviations: LA, lactic acid; Lm, *Listeria monocytogenes*

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2009; Tang et al., 2015).

In the present study, the effect of short-term lactic acid treatment on the risk of Lm was investigated in consideration of the manner of its practical use in food manufacturing processes. Since foods typically have a waiting period after LA treatment until the next processing step, the effects of the post-treatment time on the heat tolerance of Lm were also evaluated in the presence and absence of bacterial medium as a representative organic material. Further, the expression of genes related to the stress response, including *sigB*, was examined using RNA-seq and RT-PCR in order to elucidate the heat resistance mechanism of Lm following lactic acid treatment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The Lm strains used in this study were as follows: serotype 4b strain ATCC19115 (Lm19115), serotype 1/2a strain ATCC15313 (Lm15313), and serotype 1/2c strain ATCC7644 (Lm7644) purchased from the American Type Culture Collection (Manassas, VA, USA), serotype 1/2a strain MCRI-471 (Lm471) originally isolated from pork loin, and serotype 4b strain b057 (Lmb057) isolated from smoked salmon (Nakamura et al., 2004). These strains were preserved in trypticase soy broth (TSB; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 20% glycerol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at − 45 °C.

Each bacterial strain was inoculated from the frozen stock into 10 ml of TSBYE, TSB supplemented with 0.6% yeast extract (YE; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and incubated overnight at 37 °C. Bacterial solutions were diluted to 1/1000 in 50 ml tubes containing fresh TSBYE and incubated at 37 °C for 18 h. Cultures were centrifuged at 12,000 × *g*, 25 °C for 2 min and the supernatants were discarded prior to use in the following experiments.

2.2. Lactic acid treatment

Lactic acid (LA, food additive grade; Wako Pure Chemical Industries, Ltd.) was diluted to 0.5–5.0% (w/w) with TSBYE or pure water, and the diluted solutions were filter-sterilized (0.20 μm syringe filter). After preincubation at 25 °C, 20 ml of the LA solution was transferred into the tube containing the pelleted Lm cells and mixed. The cell suspension was incubated at 25 °C for up to 60 min in a thermostatic bath (Thermominder SP-12R; TITEC Corporation, Koshigaya, Saitama, Japan) (Fig. 1). A control sample was prepared using the same procedure with TSBYE or pure water instead of the LA solution. These experiments were performed in three independent trials.

2.3. Effect of time intervals following LA treatment on heat tolerance

After the treatment with 1.0% LA TSBYE solution at 25 °C for 1 min, the entire cell suspension was centrifuged at 12,000 × *g*, 25 °C for 2 min and the supernatant was discarded (Fig. 1). TSBYE was transferred into the tube containing the pelleted cells and mixed, and then incubated at 25 °C in the thermostatic bath. After incubation at 25 °C for the predetermined time intervals (0 to 360 min), the entire cell suspension was centrifuged at 12,000 × *g*, 25 °C for 2 min and the supernatant was discarded. These pelleted cells were used for the thermal treatment.

2.4. Thermal treatment

Preheated (55 °C) TSBYE was transferred into the tube containing the pelleted cells and mixed, and then incubated at 55 °C in the thermostatic bath for 10 to 30 min (Fig. 1). Three independent experiments were carried out.

2.5. Enumeration of Lm

At the predetermined time points, LA-treated or heated samples were serially diluted in TSBYE and plated onto two trypticase soy agar plates (Nissui Pharmaceutical Co., Ltd.) supplemented with 0.6% YE, respectively. Plates were incubated at 37 °C for 48 h and colonies were enumerated.

2.6. RNA extraction

After incubation at 25 °C for the predetermined time intervals, the entire cell suspension was centrifuged at 12,000 × *g*, 25 °C for 2 min and the supernatant was discarded. Pelleted cells were washed with a mixture of 2 ml of PBS and 4 ml of RNAprotect Bacteria Reagent (QIAGEN GmbH, Hilden, Germany), incubated at 25 °C for 5 min, and centrifuged at 5000 × *g*, 25 °C for 5 min prior to removal of the supernatant. One milliliter of lysozyme (Wako Pure Chemical Industries, Ltd.) solution (100 mg/ml in TE buffer) was added and the sample was incubated at 37 °C for 10 min. The entire cell suspension was transferred into a 2.0-ml tube, centrifuged at 10,000 × *g*, 25 °C for 5 min and the supernatant was discarded. Preheated (50 °C) Sepasol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) was added to the tube and mixed by pipetting. The lysate was incubated at 50 °C for 10 min and mixed by pipetting again. After a further 30 min incubation, the lysates were cooled at room temperature (RT) for 5 min. A 200-μl aliquot of chloroform was added to the tube and vortexed, and then incubated at RT for 3 min. The tube was centrifuged at 12,000 × *g*, 4 °C for 15 min,

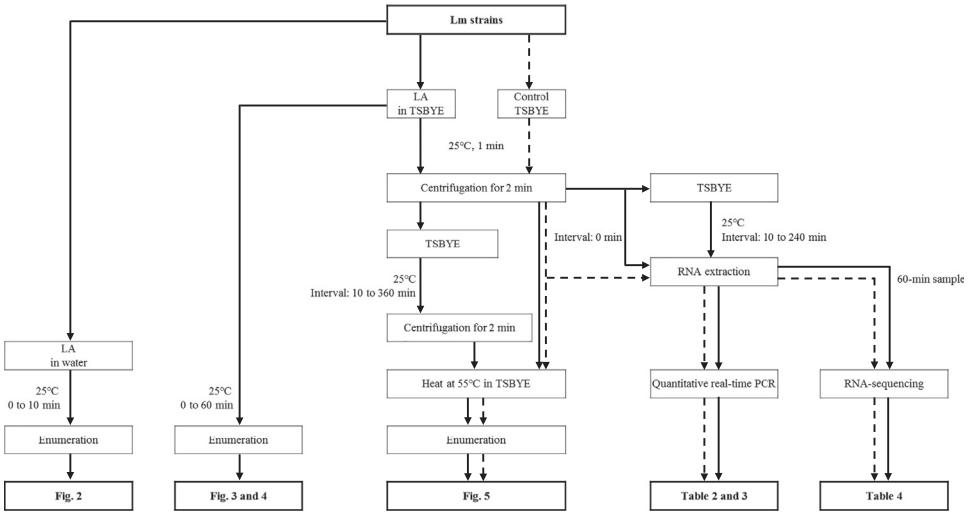

Fig. 1. Flow chart of this study.

and the aqueous layer was transferred to a fresh tube to which 500 µl of 2-propanol was added and vortexed. After incubation at RT for 10 min, the tube was centrifuged at 12,000 × *g*, 4 °C for 10 min and the supernatant was removed. A 600-µl aliquot of DEPC (diethyl pyrocarbonate)-treated water, 100 µl of 5 M NaCl and 80 µl of preheated (65 °C) 10% CTAB (cetyltrimethylammonium bromide; Calbiochem, San Diego, CA, USA)-0.7 M NaCl were added and vortexed, followed by incubation at 65 °C for 10 min. A 700-µl aliquot of chloroform-isoamyl alcohol was added to the tube and vortexed, followed by centrifugation at 12,000 × *g*, 4 °C for 10 min, and the aqueous layer was then transferred to a fresh tube to which 500 µl of 2-propanol was added. This solution was vortexed and incubated at RT for 10 min. The RNeasy Mini Kit (QIAGEN GmbH) was used to isolate bacterial RNA following the manufacturer's instructions. The RNA eluted with 30 µl of RNase-free water was used for cDNA synthesis and RNA-seq.

2.7. cDNA synthesis

The contaminating genomic DNA in the RNA was removed using the gDNA Wipeout Buffer of a Quantitect Reverse Transcription Kit (QIAGEN GmbH) according to the manufacturer's instructions. The RNA was reverse transcribed in 20 µl of total reaction volume, containing 14 µl of RNA solution, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5 × Quantiscript RT Buffer, and 1 µl of RT Prime Mix. cDNA synthesis was conducted in a thermal cycler (Thermal Cycler Dice Touch model TP350; TAKARA BIO INC., Otsu, Shiga, Japan) under the following conditions: reverse transcription at 42 °C for 15 min, enzyme inactivation at 95 °C for 3 min, cooling at 4 °C.

2.8. Quantitative real-time PCR

Gene expression of heat shock proteins in Lm in response to LA treatment was investigated using quantitative real-time PCR. Each 25-µl reaction solution was composed of 1 µl of cDNA solution, 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 3 µl (2.5 µM) of each primer and 5.5 µl of nuclease-free water. The primer sequences used to examine the gene expression of heat shock proteins in this study are listed in Table 1. These primer sets were designed using the online site: Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The specificity of each primer set was determined using melting curve analysis during real-time PCR. Quantification of target genes was carried out with a StepOnePlus Real-Time PCR System (Applied Biosystems) using the following running conditions: initial denaturation at 95 °C for 10 min and 40 cycles of denaturation at 94 °C for 15 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s. PCR accuracy was validated by melt curve analysis of the product to 95 °C from 60 °C, at temperature increments of 0.3 °C. No-template controls were included in each assay. All samples were amplified in separate wells in duplicate. Individual gene expression levels were calculated using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001). The 16S rRNA gene was used as an internal control. Three independent experiments were carried out.

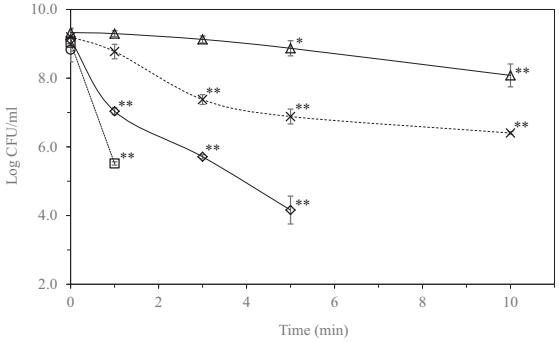

Fig. 2. Survival curves of Lm19115 exposed to a water solution of LA at various concentrations. 0.5% (△), 1.0% (×), 2.0% (◊), 3.0% (□), 5.0% (○). The bacterial cells were suspended in LA solutions at 25 °C, and the numbers were enumerated over time. Error bars represent the standard error of three independent trials. Asterisks indicate a significant difference compared to the initial number (*: $P < 0.05$, **: $P < 0.01$).

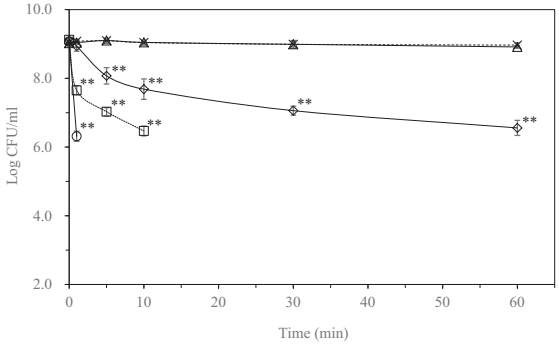

Fig. 3. Survival curves of Lm19115 exposed to a TSBYE solution of LA at various concentrations. 0.5% (△), 1.0% (×), 2.0% (◊), 3.0% (□), 5.0% (○). The bacterial cells were suspended in LA solutions at 25 °C, and the numbers were enumerated over time. Error bars represent the standard error of three independent trials. Asterisks indicate a significant difference compared to the initial number (**: $P < 0.01$).

2.9. RNA sequencing

RNA sequencing was performed one time by Macrogen Japan Corp. (Kyoto, Japan). After elimination of contaminating DNA by DNase, the quality, quantity and RNA Integrity Number (RIN) value of total RNA samples were assessed using an Agilent 2100 Bioanalyzer. Samples were confirmed for RIN values > 7. rRNA was depleted from the samples using a Ribo-Zero rRNA Removal (Bacteria) Kit (Illumina Inc., San Diego, CA, USA). cDNA libraries were created using a TruSeq RNA Sample Prep Kit v2 (Illumina Inc.). The cDNA libraries were sequenced using an Illumina HiSeq2000 instrument to perform 100 paired-end sequencing runs. The quality control of the sequenced raw reads was analyzed (FastQC v0.10.0: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Overall read quality, total bases, total reads, GC (%) and basic statistics were calculated. To reduce biases in analysis, artifacts such as low-quality reads, adaptor sequence, contaminant DNA or PCR duplicates were removed (Trimmomatic 0.32: http://www.

Table 1
Primers used for real-time PCR in this study.

Gene	Forward primer	Reverse primer	Products size (bp)
16S rRNA	AGCGTGGGGAGCAAACA	AGCACTAAGGGGCGGAAAC	91
dnaJ	TCAGGTGTTGTCCCTGGTTT	CCCAAGACAAGGTAGCGATTT	147
dnaK	TTGAAACCAACGACAGAAGGTG	CAGTAGCAGTATTAGAAGGCGGAGA	85
grpE	TGGGCGTATAACCCGATCTT	CTGTCGGCGAACAATTTGAC	131
groEL	GTGATGACTTGTCCGCCTGT	CGTGAAAGCTCCTGGTTTTG	81
groES	TCCTTCATACGTCACTTCTGTTCC	TTGTTGCAGTCGGTTCAGGT	119
clpB	CGTCGAGTTCACTTGGCATC	CAGCAGCGAGTCTTTCTAATCGT	126
clpE	CGATTGTGCGGTATTCTTTTAGTG	CGATTGTTGGGGCAGGTT	123

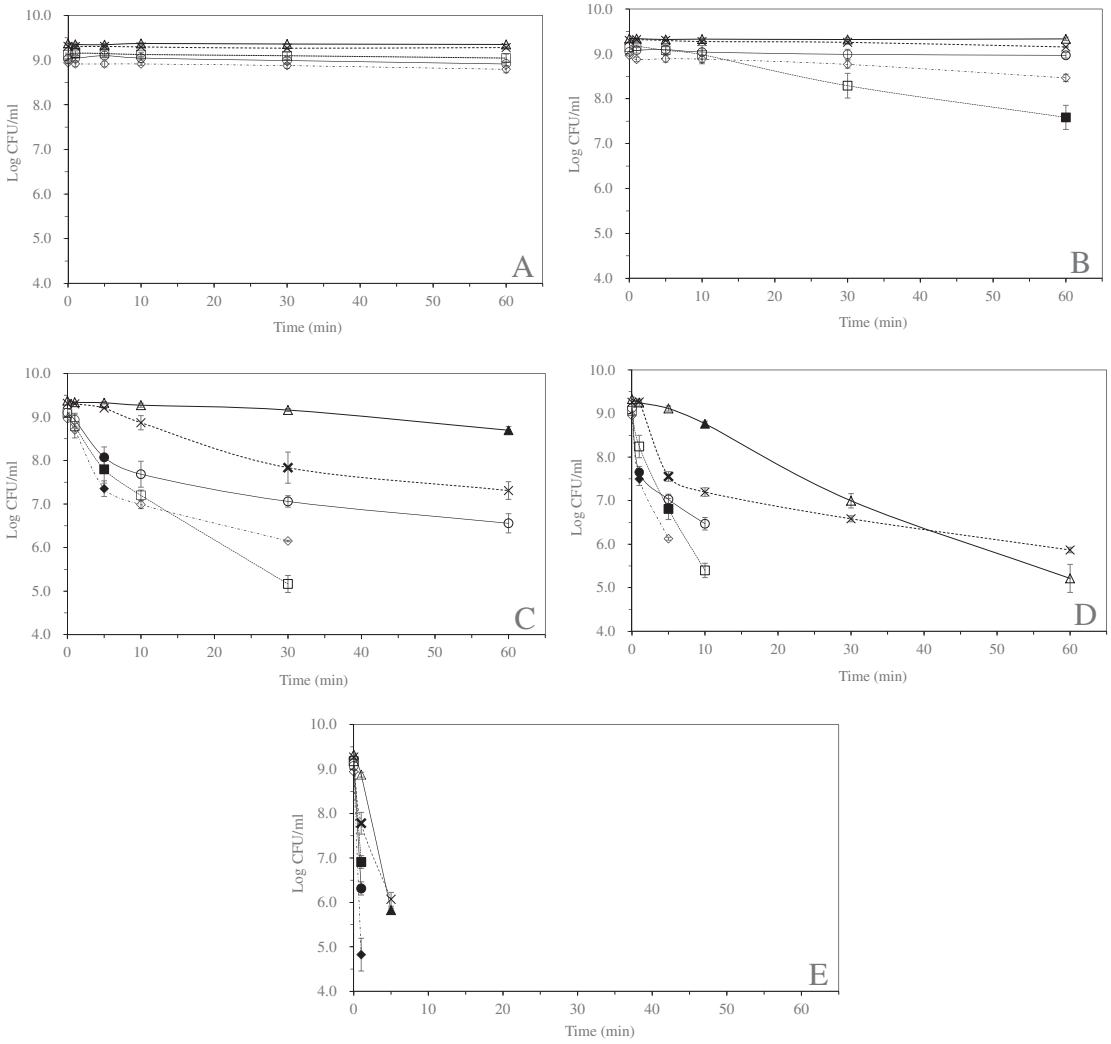

Fig. 4. Survival curves of various Lm strains in a TSBYE solution of LA at various concentrations. 0.5% (A), 1.0% (B), 2.0% (C), 3.0% (D), 5.0% (E). Lm19115 (○), Lm7644 (□), Lm15313 (◊), Lm471 (×), Lmb057 (△). The bacterial cells were suspended in LA solutions at 25 °C, and the numbers were enumerated over time. Error bars represent the standard error of three independent trials. The closed or bold symbols indicate the first point at which there is significant difference compared with the initial number ($P < 0.01$).

usadellab.org/cms/?page=trimmomatic). Trimmed reads were mapped to the reference genome (*L. monocytogenes* serotype 4b str. CLIP 80459: GCF_000026705.1) with Bowtie (http://bowtie-bio.sourceforge.net/index.shtml). RPKM (Fragments Per Kilobase of CDS per Million Mapped reads) values were used to normalize and quantify the gene expression level. Analysis of differentially expressed genes was calculated with the G option of the Cufflinks program (http://cole-trapnell-lab.github.io/cufflinks/). Contrast with the EGD-e loci (lmo genes) was performed using the NCBI nucleotide database (Clip80459: NC_012488.1 and EGD-e: NC_003210.1) (National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/), Uniprot (http://www.uniprot.org/) and the alignment analysis tool ClustalW of the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/searches-j.html).

2.10. Statistical analysis

Changes in Lm numbers at individual time points compared to initial numbers in the water and TSBYE solutions of LA were analyzed by one-factor ANOVA and Tukey's test. Differences in the survival curves for Lm following heat treatment were analyzed using two-factor (interval time after LA treatment and heating) ANOVA and Tukey's test. Differences in gene expression levels of heat shock proteins were analyzed using one-factor (time after LA treatment) ANOVA and Tukey's test.

3. Results

3.1. Effect of LA treatment on Lm survival

Survival curves for Lm19115 in water solutions of LA were generated (Fig. 2). Following exposure to the 0.5% LA solution, Lm19115 numbers were decreased significantly at 5 min (Fig. 2), and a 1.2 Log CFU/ml decrease was observed at 10 min. The number of Lm19115 in the 1.0% LA solution decreased significantly by 1.8, 2.3 and 2.8 Log CFU/ml after 3, 5 and 10 min, respectively. In the 2.0 and 3.0% LA solutions, Lm19115 numbers decreased by 2.1 and 3.5 Log CFU/ml, respectively, in 1 min. Lm19115 exposed to the 5.0% LA solution were not detected after 1 min.

The survival curves of Lm in TSBYE (exposure to organic matter) solutions of LA differed obviously from those exposed to the water solutions of LA: Lm was more resistant in the TSBYE solutions of LA (Fig. 3). For 0.5 and 1.0% LA in TSBYE, Lm19115 numbers did not change over the 60-min time course (Fig. 3). The numbers of Lm19115 in the 2.0, 3.0 and 5.0% LA solutions decreased significantly by 1.0, 1.5 and 2.8 Log CFU/ml in 5 min, 1 min and 1 min, respectively.

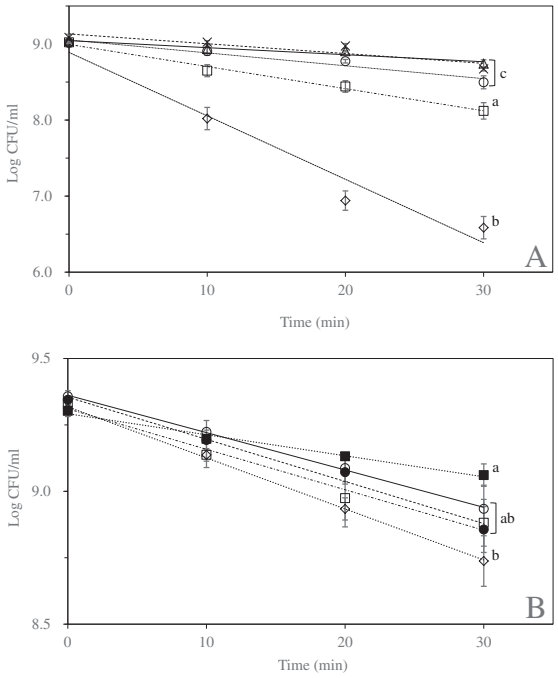

Fig. 5. Survival curves of Lm at 55 °C. Lm strains were treated with LA, and heat resistance was assessed after various recovery times. In Lm19115 (A), the time intervals in TSBYE after LA treatment were 0 min (◊), 60 min (○), 120 min (△), and 360 min (×); the non-treated control (□). In Lmb057 (B), the time intervals in TSBYE after LA treatment were 0 min (◊), 30 min (■), 60 min (○), 90 min (●), and the control was (□). Different letters within curves indicate significant differences ($P < 0.01$). Error bars represent the standard error of three independent trials.

3.2. Differences in LA susceptibility of Lm strains

Survival curves of Lm strains exposed to LA in TSBYE were compared (Fig. 4). The numbers of Lm15313, Lm471 and Lmb057 in the 0.5 and 1.0% LA solution did not show significant changes (Fig. 4A, B); these strains were comparatively resistant to LA. In contrast, the number of Lm7644 in the 1.0% LA solution decreased significantly by 1.6 Log CFU/ml after 60 min (Fig. 4B). Lmb057 numbers in the 2.0% LA solution decreased by 0.7 Log CFU/ml after 60 min (Fig. 4C). Although the degree of reduction varied depending on the strain, all Lm strains were significantly decreased within 10 min in the 3.0% LA solution (Fig. 4D). In the 5.0% LA solution, rapid decreases were observed in all Lm strains (Fig. 4E). The number of Lmb057 decreased by 3.5 Log CFU/ml after 5 min.

3.3. Changes in heat resistance

Survival curves for Lm19115 at 55 °C were generated (Fig. 5A). The numbers of Lm held for 0, 60, 120 and 360 min after LA treatment decreased by 2.4, 0.5, 0.3, and 0.4 Log CFU/ml after 30-min heat treatment, while the non-treated control sample decreased by 0.9 Log CFU/ml; the calculated D-values ± standard error were 12.1 ± 0.8, 65.7 ± 11.6, 124.8 ± 28.5, 82.4 ± 10.6, and 35.4 ± 3.5 min, respectively. Fold changes compared to the control were − 2.9 ± 0.2, 1.8 ± 0.2, 3.4 ± 0.5 and 2.5 ± 0.5, respectively. The heat resistance of Lm19115 held in TSBYE at 25 °C for 60, 120, and 360 min increased significantly compared to the control and that immediately after the LA treatment ($P < 0.01$).

Survival curves for Lmb057 at 55 °C were generated (Fig. 5B). Calculated D-values of non-treated Lm and those held for 0, 30, 60 and 90 min following LA treatment were 71.3 ± 14.7, 54.5 ± 8.7, 128.4 ± 10.7, 78.4 ± 16.3 and 69.3 ± 16.1 min, respectively. The respective fold changes compared to the control were − 1.3 ± 0.1, 1.9 ± 0.2, 1.3 ± 0.2 and 1.0 ± 0.0. The heat resistance of the 30 min sample increased significantly compared to the 0 min sample ($P < 0.01$); although the resistance of Lmb057 was not further enhanced with longer time intervals.

3.4. Changes in gene expression of heat shock proteins

Gene transcription of Lm19115 heat shock proteins was examined using quantitative real-time PCR (Table 2). Expression levels of *dnaJ*, *dnaK*, *grpE*, *groEL* and *groES* did not significantly differ compared to the control. The genes *clpB* and *clpE* showed a significant increase of 11.5 and 21.0-fold at 60 min compared to the control and then decreased at 3 h, respectively ($P < 0.05$). The expression of *clpE* was also significantly upregulated at 30 and 90 min.

The relative gene expression of Lmb057 heat shock proteins is shown in Table 3. Transcription of the genes except for *dnaJ* significantly increased at 10 min compared to 0 min and then decreased over 110 min ($P < 0.05$). Gene expression, especially that of *clpE*, was strongly down-regulated at 120 min. There were no significant differences between control and 10 min except for *dnaK*, *groEL*, and *clpB*.

3.5. RNA sequencing (RNA-seq)

RNA-seq analysis was conducted to identify genes besides *clpB*, and *clpE* that are associated with Lm19115 heat resistance. The data were registered with the DNA Data Bank of Japan Sequence Read Archive (DRA; http://trace.ddbj.nig.ac.jp/dra/index.html); a member of International Nucleotide Sequence Database Collaboration (INSDC). The accession number is DRA005721. Differentially expressed gene analysis was performed using samples with the 60 min interval after LA treatment and the non-treated control. A total of 2782 genes were mapped, and 1580 genes showed a ≥ 2-fold change. The expression of 875 genes was increased.

Heat-shock genes not examined by quantitative real-time PCR were determined by RNA-seq. Three class III heat-shock genes (*ctsR*, *mcsB*, and *clpP*) were upregulated (Table 4). Gene expression of *ctsR*, *mcsB*,

Table 2
Changes in the gene expression of Lm19115 heat shock proteins.

Gene	Control	Interval of time after lactic acid treatment (min)						
		0	10	30	60	90	120	240
dnaJ	1 ^{ab}	1.1 ± 0.2 ^{ab}	− 1.4 ± 0.2 ^{a}	2.0 ± 0.7 ^{ab}	2.2 ± 0.3 ^{b}	1.5 ± 0.2 ^{ab}	1.9 ± 0.1 ^{ab}	− 1.0 ± 0.1 ^{ab}
dnaK	1	− 1.1 ± 0.1	− 1.3 ± 0.2	2.4 ± 0.7	2.7 ± 0.4	2.2 ± 0.3	2.2 ± 0.7	− 1.1 ± 0.1
grpE	1 ^{ab}	− 1.2 ± 0.1 ^{a}	− 1.1 ± 0.2 ^{ab}	2.4 ± 0.3 ^{ab}	2.6 ± 0.6 ^{b}	2.4 ± 0.3 ^{ab}	2.3 ± 0.6 ^{ab}	− 1.2 ± 0.1 ^{a}
groEL	1	1.1 ± 0.2	− 1.7 ± 0.1	− 1.0 ± 0.2	1.3 ± 0.2	1.6 ± 0.4	1.2 ± 0.3	− 1.2 ± 0.1
groES	1	1.2 ± 0.2	− 1.6 ± 0.1	1.9 ± 0.1	1.6 ± 0.3	1.6 ± 0.2	1.4 ± 0.5	− 1.2 ± 0.2
clpB	1 ^{a}	1.2 ± 0.3 ^{a}	1.9 ± 0.3 ^{a}	6.6 ± 2.0 ^{ab}	11.5 ± 2.6 ^{b}	7.0 ± 1.5 ^{ab}	6.7 ± 1.2 ^{a}	1.8 ± 0.1 ^{a}
clpE	1 ^{a}	− 1.3 ± 0.1 ^{a}	4.8 ± 0.4 ^{ab}	13.2 ± 3.6 ^{bc}	21.0 ± 4.0 ^{c}	13.9 ± 2.0 ^{bc}	8.1 ± 0.6 ^{ab}	1.7 ± 0.3 ^{a}

All measurement were done in triplicate with replication, and all values are means ± standard error.
Mean values in the same line followed by different superscripts are significantly different ($P < 0.05$).

Table 3
Changes in the gene expression of Lmb057 heat shock proteins.

Gene	Control	Interval of time after lactic acid treatment (min)					
		0	10	30	60	90	120
dnaJ	1	1.1 ± 0.6	8.5 ± 3.5	8.7 ± 1.6	4.9 ± 2.1	3.8 ± 0.8	1.2 ± 0.1
dnaK	1	−3.8 ± 0.1	3.1 ± 0.7 [a]	−1.3 ± 0.1	−1.8 ± 0.2	−3.3 ± 0.0	−11.1 ± 0.0
grpE	1 [ab]	−12.2 ± 0.0 [a]	1.8 ± 0.5 [b]	−1.9 ± 0.1 [a]	−3.2 ± 0.1 [a]	−6.1 ± 0.0 [a]	−18.0 ± 0.0 [a]
groEL	1	−2.1 ± 0.2	2.4 ± 0.5 [a]	−1.4 ± 0.2	−1.5 ± 0.1	−3.4 ± 0.0	−6.8 ± 0.1
groES	1	−7.9 ± 0.0 [a]	1.4 ± 0.3	−3.2 ± 0.1 [a]	−3.5 ± 0.1 [a]	−6.0 ± 0.1 [a]	−11.6 ± 0.1 [a]
clpB	1 [a]	−7.1 ± 0.1 [b]	1.7 ± 0.3 [c]	−2.2 ± 0.1 [ab]	−3.1 ± 0.1 [b]	−8.2 ± 0.0 [b]	−27.0 ± 0.0 [b]
clpE	1 [ac]	−4.0 ± 0.1 [ab]	1.6 ± 0.3 [c]	−3.0 ± 0.1 [ab]	−2.5 ± 0.3 [ab]	−13.0 ± 0.0 [b]	−110.0 ± 0.0 [b]

All measurement were done in triplicate with replication, and all values are means ± standard error.
Mean values in the same line followed by different superscripts are significantly different ($P < 0.05$).

and *clpP* was upregulated by 2.4, 2.1, and 11.7-fold, respectively.

The class II stress genes regulated by the alternative sigma factor SigB (listed in Table 4) were reportedly upregulated in response to 48 °C exposure for 3 min (van der Veen et al., 2007). The SigB-regulated genes except for lmo2386 were downregulated in this study. Expression of the membrane protein gene lmo2386 was upregulated by 3.9-fold.

The function of the SOS response is DNA repair and synthesis. The Lm SOS response regulon consists of 29 genes, and RecA, an activator of the SOS response, is important for stress resistance (van der Veen et al., 2010). Thus, RecA and the SOS response factors activated by RecA are important for stress survival. The present study showed that 11 genes involved in the SOS response and DNA repair were upregulated, including *recA* (Table 4).

The other genes listed in Table 4 were also reportedly associated with bacterial resistance. Ten putative heat resistance-related genes were upregulated. The expression of these genes increased by 2.3 to 8.0-fold in this study.

4. Discussion

The results indicate that Lm could be reduced significantly by ≥ 2.0% LA within 5 min. However, components in the TSBYE medium appeared to decrease the effect of LA. This finding suggests the possibility that LA is less effective for Lm harbored in minute food crevices or in residues on food processing equipment at manufacturing sites. Further, the effect of LA is dependent on the strain of Lm, and a LA concentration of 3.0% at a minimum is required for effective Lm disinfection; although, a concentration of 2% is consistent with EU regulations concerning the use of LA to reduce microbiological surface contamination on bovine carcasses (Official Journal of the European Union, 2013). Previous reports also revealed that the responses to stressors such as salt, acids, and heat vary depending on the strain of Lm (Faleiro et al., 2003; Francis and O'Beirne, 2005; Lunden et al., 2008; Schirmer et al., 2014; Sorqvist, 1994).

A heating test was carried out assuming Lm survival after LA treatment. Time intervals were set in reference to the wait times of food manufacturing processes. Previous reports showed that the pH of acid-treated meat surfaces increased with time, even at low temperatures (Anderson and Marshall, 1990; Gonzalez-Fandos and Dominguez, 2006; van Netten et al., 1997). Therefore, the time interval in this test was carried out in fresh medium containing organic materials. Lm was more vulnerable to heat treatment immediately following LA treatment. A synergistic effect is expected with heat treatment performed immediately after LA treatment. However, the results suggested that the heat resistance of some Lm strains could increase in proportion to the time interval. The wait times at food production sites may enhance the heat resistance of Lm, depending on factors such as nutrients available in the environment, time interval, temperature, and the strains. Although Lm in TSBYE was held at 25 °C after LA treatment in this study, gains in heat resistance appeared to be more difficult when the organisms were refrigerated with ice in preliminary experiments (data not shown). Thus, the cold chain throughout manufacturing processes after LA treatment is obviously important in preventing the acquisition of resistance in bacteria. Lm strains showing enhanced resistance after stress such as acid treatment would be candidate persistent strains (Nakamura et al., 2013). To investigate responsible mechanisms of the enhanced resistance at the gene level, transcription of heat shock proteins was examined using quantitative real-time PCR. The results for Lm19115 suggest that ClpB and ClpE are involved in enhancing the heat resistance of Lm19115. The genes *clpB* and *clpE* are reported to be expressed in the heat shock response (van der Veen et al., 2007). A *clpB* deficient mutant showed a 1000-fold reduction in CFU after 20 min exposure to 55 °C compared to the wild-type strain (van der Veen et al., 2009). ClpE, a member of the Clp family of ATP-dependent proteases, is also required for stress survival and intracellular growth (Nair et al., 2000). Lmb057, which is more heat-resistant than Lm19115, showed recovery of gene expression levels except for *dnaJ* at 10 min, and a significant difference in heat resistance was observed between 0 and 30 min. This data indicates that heat resistance at 55 °C was enhanced during the subsequent 10–30 min, when the bacteria were recovering from LA treatment in TSBYE. Differences in gene upregulation in Lm19115 and Lmb057 would result in differences in innate sensitivity, as Lmb057 was highly resistant to LA and heat treatment.

RNA-seq revealed the upregulation of genes *ctsR*, *mcsB* and *clpP*. ClpP acts as protease to remove damaged and/or misfolded proteins, and plays a role in heat-induced growth arrest. A mutant lacking ClpP was more sensitive to the inhibitory effects of heat, salt, and oxidative stress than the isogenic wild-type strain of *Streptococcus agalactiae* (Nair et al., 2003). Arginine residues of aberrant proteins are phosphorylated by McsB, and the protein is transferred to ClpP by ClpC, which is also initiated by McsB. Subsequently, ClpP can degrade the target proteins (Trentini et al., 2016). Furthermore, McsB appears to act as a suppressor of CtsR, which is a down-regulator of *clpC*, *clpE*, *clpP*, and *clpX* (Nair et al., 2000; Tao et al., 2012). These facts suggest that the increased expression of *clpP* and *mcsB* was involved in the heat resistance of Lm19115 in this study.

Several reports have demonstrated that SigB is an important factor in heat resistance (Ait-Ouazzou et al., 2012; Somolinos et al., 2010; van Schaik et al., 2004; Volker et al., 1999). However, the SigB-regulated genes except for lmo2386 were downregulated in the present study. This is unlikely to be due to the loss-of-function of SigB since the deduced amino acid sequences indicate the presence of only a single point mutation (Tyr216Phe) compared to the database of EGD-e. Thus, the acquisition of heat resistance by Lm19115 may possibly be due to another mechanism that differs from the heat shock response. Ait-Ouazzou et al. (2012) showed that the absence of *sigB* did not decrease the heat resistance of Lm at pH 4.0, in contrast to that observed at pH 7.0 and 5.5. The results suggest that the low pH induces some substances that support heat resistance, independent of SigB. Ferreira et al. (2001) demonstrated that acid shock (at pH 4.5 for 1 h) could

Table 4
Differential gene expression analysis of Lm19115 at 60 min following LA treatment compared to control.

Gene locus	EGD-e locus	Gene designation	Protein ID	Description of product	Fold-change
ClassIII heat-shock genes					
LM4B_RS01225	lmo0229	*ctsR*	WP_003726421.1	CtsR family transcriptional regulator	**2.4**
LM4B_RS01235	lmo0231	*mcsB*	WP_003726423.1	Protein arginine kinase	**2.1**
LM4B_RS12415	lmo2468	*clpP*	WP_003722600.1	ATP-dependent Clp protease proteolytic subunit	**11.7**
ClassII stress genes (SigB regulated)					
LM4B_RS01055	lmo0211	*ctc*	WP_003722740.1	50S ribosomal protein L25/general stress protein Ctc	− 3.3
LM4B_RS03955	lmo0781		WP_003721898.1	Similar to mannose-specific phosphotransferase system (PTS) component IID	− 34.9
LM4B_RS03960	lmo0782		WP_003724495.1	PTS mannose/fructose/sorbose transporter subunit IIC	− 23.5
LM4B_RS03965	lmo0783		WP_003721900.1	PTS fructose transporter subunit IIB	− 21.1
LM4B_RS04025	lmo0794		WP_003726297.1	Hypothetical protein	− 2.4
LM4B_RS04465	lmo0880		WP_012681191.1	Peptidoglycan linked protein (LPXTG)	− 2.3
LM4B_RS04650	lmo0911		WP_003724838.1	Hypothetical protein	− 4.1
LM4B_RS04870	lmo0956		WP_003726858.1	Similar to *N*-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	− 8.5
LM4B_RS04875	lmo0957	*nagB*	WP_003727073.1	Glucosamine-6-phosphate deaminase	− 5.9
LM4B_RS04880	lmo0958		WP_003722779.1	GntR family transcriptional regulator	− 5.9
LM4B_RS07155	lmo1425	*opuCD*	WP_003725369.1	Amino acid ABC transporter permease	− 37.7
LM4B_RS07160	lmo1426	*opuCC*	WP_003721931.1	Carnitine transport-binding protein OpuCC	− 19.5
LM4B_RS07165	lmo1427	*opuCB*	WP_003725370.1	Carnitine transport permease OpuCB	− 10.4
LM4B_RS07170	lmo1428	*opuCA*	WP_003725371.1	Glycine/betaine ABC transporter ATP-binding protein	− 3.6
LM4B_RS07720	lmo1538	*glpK*	WP_003726648.1	Glycerol kinase	− 10.6
LM4B_RS07930	lmo1580		WP_003725993.1	Universal stress protein UspA	− 69.6
LM4B_RS08640	lmo1694		WP_003728261.1	Epimerase	− 4.1
LM4B_RS09605	lmo1883		WP_003728270.1	Chitinase	− 2.5
LM4B_RS10550	lmo2067		WP_003728858.1	Choloylglycine hydrolase	− 4.0
LM4B_RS12005	lmo2386		WP_003722412.1	Membrane protein	**3.9**
LM4B_RS12070	lmo2398	*ltrC*	WP_003725592.1	Phosphatidylglycerophosphatase A	− 5.7
LM4B_RS12930	lmo2572		WP_003726114.1	Diacylglycerol kinase	− 14.0
LM4B_RS13110	lmo2602		WP_003730782.1	Methyltransferase	− 40.6
SOS response and DNA repair					
LM4B_RS00810	lmo0158		WP_012681021.1	Sugar phosphate phosphatase	**2.6**
LM4B_RS06545	lmo1302	*lexA*	WP_003723438.1	LexA repressor	**4.0**
LM4B_RS07020	lmo1398	*recA*	WP_003725961.1	DNA recombination/repair protein RecA	**4.9**
LM4B_RS10040	lmo1975	*dinB*	WP_003725867.1	DNA polymerase IV	**3.3**
LM4B_RS12515	lmo2488	*uvrA*	WP_003725420.1	Excinuclease ABC subunit A	**2.3**
LM4B_RS12520	lmo2489	*uvrB*	WP_003725421.1	UvrABC system protein B	**2.2**
LM4B_RS13505	lmo2675		WP_003724982.1	Hypothetical protein	− 8.9
LM4B_RS13510	lmo2676		WP_003724983.1	Type VI secretion protein ImpB	− 5.9
LM4B_RS14280	lmo2828		WP_003727591.1	Hypothetical protein	− 3.1
LM4B_RS03040	lmo0588		WP_003725898.1	Deoxyribodipyrimidine photo-lyase	**2.1**
LM4B_RS04730	lmo0928		WP_003724850.1	DNA-3-methyladenine glycosylase	**3.4**
LM4B_RS06180	lmo1234	*uvrC*	WP_003726544.1	UvrABC system protein C	**2.4**
LM4B_RS06870	lmo1368	*recN*	WP_003738883.1	DNA repair protein RecN	− 2.2
LM4B_RS07275	lmo1449	*nfo*	WP_012681296.1	Endonuclease	**2.5**
LM4B_RS12690	lmo2523		WP_003727895.1	Single-strand-binding protein	**2.7**
Genes of putative heat resistance related					
LM4B_RS00010	lmo0002	*dnaN*	WP_003725616.1	DNA polymerase III subunit beta	**8.0**
LM4B_RS01105	lmo0220	*ftsH*	WP_012681032.1	Cell division protein FtsH	**4.7**
LM4B_RS01120	lmo0223	*cysK*	WP_003725748.1	Cysteine synthase A	**2.7**
LM4B_RS07280	lmo1450	*cshB*	WP_003721956.1	DEAD/DEAH box family ATP-dependent RNA helicase	**3.4**
LM4B_RS07465	lmo1487		WP_003726533.1	Phosphohydrolase	**6.3**
LM4B_RS07505	lmo1495		WP_003734326.1	Hypothetical protein	**2.3**
LM4B_RS07510	lmo1496	*greA*	WP_003722004.1	Transcription elongation factor GreA	**4.7**
LM4B_RS08315	lmo1657	*tsf*	WP_003726264.1	Elongation factor Ts	**5.3**
LM4B_RS10440	lmo2045		WP_003726129.1	Hypothetical protein	**5.6**
LM4B_RS12675	lmo2520	*menC*	WP_003727896.1	*O*-succinylbenzoate synthase	**4.3**

The value of equal to or > 2-fold increase is given in bold type (plus value only).

endow marked acid resistance even in the *sigB* deficient strain. Moreover, the study suggested the existence of both a SigB-dependent mechanism and a pH-dependent mechanism to describe the acid resistance of Lm. In this study, the pH-dependent mechanism by acid shock could presumably contribute to the enhanced heat resistance.

van der Veen et al. (2010) showed that a mutant lacking RecA was less resistant to heat, H_2O_2, and acid exposure than the wild-type. The mutant showed approximately 1000-fold fewer cell counts after 1 h exposure to 55 °C. Upregulation of 4 (*recA*, *dinB*, *uvrA*, and *uvrB*) of the 11 genes after LA treatment is supported by a previous report (van der Veen et al., 2007). These results suggest that the *recA*, *dinB*, *uvrA* and *uvrB* genes are involved in promoting the heat resistance of Lm19115.

DnaN is a subunit of the DNA polymerase III repair and stress protein complex (Villarroya et al., 1998). Agoston et al. (2009) found that DnaN was upregulated at 60 °C. van der Veen et al. (2009) observed that several mutant strains lacking the lmo0220, lmo1487, lmo1495, lmo2045 or lmo2520 genes were significantly more sensitive to heat stress at 55 °C than the wild-type. The mutant lacking lmo1487 was reduced by nearly 1000-fold compared to the wild-type after heating at 55 °C for 20 min. lmo0223, lmo1496 and lmo1657, genes which code for cysteine synthase A, transcription elongation factor GreA and elongation factor Ts, respectively, were also upregulated by heat

treatment at 55 °C (Guevara et al., 2015). GreA is an essential factor in the RNA polymerase elongation complex. Li et al. (2012) reported suppression of the aggregation of several substrate proteins under heat shock conditions and promotion of the refolding of denatured proteins as an additional chaperone function of GreA in *Escherichia coli*. The lmo1450 (*cshA*) mutant was more sensitive to heating at 42.5 °C (Markkula et al., 2012). These reports suggest that the 10 genes were pivotal for the survival of Lm19115 at high temperatures.

RNA-seq analysis suggested that several proteins are involved in strengthening the heat resistance of Lm19115. However, these proteins may not be class II heat-shock proteins, which are known to support heat resistance. The contributory proteins seem to be several chaperones and SOS proteins. The other genes listed in Table 4 may also encode heat resistance factors, as they were expressed only after exposure to a lethal high temperature. However, these results are based on mRNA transcription levels and not on protein expression levels. We suggest that in the future, it is necessary to verify which proteins are involved in this phenomenon using gene targeting modification technology. Also, further investigation into the generalizability of the gene expression changes observed in Lm19115 to other Lm strains is required.

In conclusion, this study reconfirmed that LA treatment is effective for inactivating Lm. However, the present data also revealed that the heat resistance of Lm was remarkably enhanced after acid treatment depending on the recovery time in the presence of organic matter. RNA-seq and RT-PCR analyses demonstrated that several chaperones, SOS proteins and products of genes associated with heat resistance are involved in the mechanism. Although hurdle technology is a well-recognized concept in food production sites and slaughterhouses, the findings of the present study highlight the need to pay attention to the combination of acid treatment and subsequent manufacturing processes.

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