BIODIVERSITAS
Volume 25, Number 6, June 2024
Pages: 2494-2499

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250618

Analysis of heat-shock protein genes and their expression in *Lactiplantibacillus plantarum* SU-KC1a

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Manuscript received: 26 April 2024. Revision accepted: 12 June 2024.

Abstract. *Julyanto CMP, Dosan R, Victor H, Sugata M, Tan TJ. 2024. Analysis of heat-shock protein genes and their expression in* Lactiplantibacillus plantarum *SU-KC1a. Biodiversitas 25: 2494-2499. Lactiplantibacillus plantarum* is lactic acid bacteria commonly used as probiotics. Throughout the manufacturing and processing stages for incorporation into functional food products, probiotics encounter different stress conditions, including heat stress, which can affect their viability. In this study, the ability of *L. plantarum* SU-KC1a to withstand heat stress conditions was assessed by measuring cell viability after incubation at 37, 42, 47, and 52°C for one hour. Additionally, the growth of SU-KC1a in liquid cultures under various high temperatures was monitored hourly for three hours. Total RNA was extracted from each culture, converted into cDNA, and amplified using *hsp3* gene-specific primers. The results indicated a slower growth rate of SU-KC1a at higher temperatures, with 42°C as the maximum temperature supporting growth. Visualization of *hsp3* amplicon showed increased intensity following incubation at higher temperatures, indicating upregulation of *hsp3* gene expression. To identify the presence of heat stress-related genes, whole genome sequence annotation data of SU-KC1a was compared to that of *L. plantarum* SK151, F75, and WCFS1. *Lactiplantibacillus plantarum* was found to possess two systems involved in protein repair in response to heat stress damage: chaperone and protease. In summary, *L. plantarum* SU-KC1a harbors heat shock protein genes, and their expression is upregulated following exposure to high temperatures.

Keywords: Comparative analysis, gene annotation, *hsp*, probiotics, reverse transcription

INTRODUCTION

Probiotics are live microorganisms that, when consumed in adequate amounts, confer a beneficial health effect on the host (WHO 2001). Probiotics cover various genera of bacteria, including *Lactobacillus* and *Bifidobacterium. Lactiplantibacillus plantarum*, previously known as *Lactobacillus plantarum*, is a commonly used probiotic in the food industry. Different strains of *L. plantarum* have been applied as a starter culture for numerous fermented food products, e.g., *L. plantarum* AJ11 in table olive, *L. plantarum* LBP-K10 in kimchi, and *L. plantarum* K4 in sourdough (Liu et al. 2017; Yu et al. 2021). During food processing, handling, and storage, probiotics might encounter various stress conditions, such as oxidative stress, heat stress, and low pH, leading to low viability. Meanwhile, to provide health benefits, probiotics need to reach a certain amount in the digestive tract (Butel 2014; Seddik et al. 2017). Hence, stress tolerance is an important trait for probiotics.

Lactiplantibacillus plantarum, a gram-positive bacterium with a rod-shaped morphology, lacks spores and thrives in microaerophilic conditions. In response to environmental stressors, *L. plantarum* employ various molecular mechanisms to adapt, survive, and resume growth. Lactobacilli utilize intricate sensory systems to monitor both intra- and extracellular environments, facilitating their ability to navigate changes in their surroundings. Signal transduction in bacteria primarily occurs through two major pathways: the one-component system (OCS), where both sensory and output signals reside within the same polypeptide, and the two-component system (TCS), which involves distinct proteins for signal input and output. Bacteria employ multiple strategies, including the detection of temperature fluctuations through mechanisms such as protein denaturation and direct sensing via thermosensory structures like proteins, lipids, DNA, and RNA. Activation of signal transduction pathways follows either route. Thermosensors in lactobacilli modulate the expression of heat stress-related genes such as *clp* and *hsp*, while diverse chaperone systems (i.e. *hsp70*, *hsp60*) and the *Clp* family of proteases aid in stress adaptation. Furthermore, *L. plantarum* harbors a small heat shock protein (sHSP) family, crucial for maintaining membrane integrity, which is conserved among lactic acid bacteria (Papadimitriou et al. 2016).

Small heat shock proteins (sHSP) are ATP-independent chaperones with small molecular weight (12-43 kDa); they adhere to unfolded protein to assist protein folding, preventing misfolding and aggregation. In addition, the protective roles of sHSPs under stress conditions are closely related to protein homeostasis (Arena et al. 2019). Gene regulation of sHSPs is upregulated by the accumulation of denatured protein in the cytoplasm. Genetic differences between species generally cause heat shock tolerance characteristics in microorganisms. *L.*

plantarum might have a better tolerance to heat stress than other lactobacilli because, unlike other lactobacilli with a single heat shock protein (HSP), *L. plantarum* has a distinctive number of small heat shock proteins (sHSP). At least three sHSPs (*hsp*1/*hsp*18.5, *hsp*2/*hsp*18.55, and *hsp*3/*hsp*19.3) have been identified in *L. plantarum*. According to Capozzi et al. (2011), the *hsp*2 gene influences cell morphology and plasma membrane fluidity, while *hsp*1 and *hsp*3 are more relevant to stress conditions such as extreme temperature, gastric acid, and oxidative stress. Specifically, *hsp*1 is responsible for cryotolerance, and *hsp*3 is more relevant to heat shock adaptation (Ferrando et al. 2016; Arena et al. 2019; Longo et al. 2021).

In 2020, *Lactiplantibacillus plantarum* SU-KC1a was successfully isolated from human breast milk at the Department of Biology, Universitas Pelita Harapan. Considering its potential as a starter culture in food industries, the present study was carried out to examine the heat tolerance and *hsp*3 expression in *L. plantarum* SU-KC1a after exposing the bacterial strain to high temperatures.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The bacterial strain *L. plantarum* SU-KC1a used in this study was originated from human breast milk in Indonesia. The culture was cryopreserved and stored at 4°C when not in use. For this study, it was cultured at 37°C for 48 hours in De Man Rogosa Sharpe (Liofilchem. Italy) agar under microaerophilic conditions.

Heat shock tolerance assessment

The assessment of heat stress tolerance was carried out according to Ma et al. (2021). The *L. plantarum* SU-KC1a strain was grown in MRS broth overnight. The liquid culture was transferred (1%) to fresh MRS broth and allowed to grow at 37℃ until the culture reached exponential phase (OD600=0.6−0.8). Subsequently, the culture was placed equally in four individual vials. Each vial was incubated under different heat stress conditions (37℃, 42℃, 47℃, and 52℃) for one hour. The number of viable cells (CFU/mL) before and after the heat treatment was determined by spread-plating serial-diluted cultures on MRS agar. The plates were incubated at 37℃ for 48 hours under microaerophilic conditions. The following equation calculated viable cell numbers (Cappuccino and Welsh 2019) and cell survival rate (Lin et al. 2020).

Viable cell numbers (CFU/mL) = (total number of single colonies on agar x dilution factor)/volume of spread-plated culture

Survival rate (%) = (number of cell count after treatment/ number of initial cell count) × 100%

Growth under heat stress conditions

The growth of *L. plantarum* SU-KC1a under different heat stress conditions was measured based on the liquid cultures' optical density (OD). The OD measurement can accurately determine the number of viable cells in liquid culture under heat stress. The heat might cause protein misfolding, leading to lysis; lysed cells could not be detected in OD measurement (Noor 2015). Cultures from solid media were inoculated to MRS broth and incubated overnight at 37℃ under microaerophilic conditions. The overnight cultures (1%) were then transferred to fresh MRS broth in four individual vials and allowed to grow at 37℃, 40℃, and 42℃ for 4 hours. The optical density of the liquid cultures was observed hourly for three hours. Next, one mL of each culture was centrifugated at 8.000×g for 3 min, then the supernatant was discarded, and the pellets were resuspended in 1 mL of phosphate buffer saline (PBS). Subsequently, the optical density of the resuspended cells was measured at 600 nm.

Primer design

The bacterial strain *L. plantarum* SU-KC1a and the reference species, *L. plantarum* WCFS1 (GenBank: AL935263.2), had an identical sequence of *hsp3* gene (5'-TTACTGAATTTCAATATGATGGGTATCCGCAGCTG CCTTCTTTGGCAAAGTTAACTGCAACACACCATTT TCATATCGTGCTTCGATCTTGTCAACATCAACGTC GGGTAAAGAATATTGCCGGCCAAAGCGACCAGTT TGACGTTCACTCGCGATAATATTACCATCTTTATC GCTTTCATCACTAATACTATCGCGTTTTACAGCAA TCGATAATGTGCCATCCCGATACTTCAAGGCAATA TTCTGTTTATCGATTCCAGGAATATCGACTTTCATC GTGTACTGATCGTCATTTTCTTTAATATCAGTCTTT AGGACCGACCCATGACTACCAGCATTTAAGAATG TCCGACCGAATCCATTAACCAAATCGTCCATCTTA GTCCAGTCATTCAACCGATCAAATAAATCATTGTG CCAATTCATCATATCGTTAGCCAT-3') in their genome sequences. The primer sequence was determined based on the whole genome sequence of *L. plantarum* SU-KC1a. In addition, the primers were designed on the flanking region of the targeted sequence (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). The forward and reverse primer sequences are 5'-TGTCAACATCAACGTCGGGTAA-3' and 5'-CGTAGTCATGGGTCGGTCCT-3', respectively. Therefore, to ensure specificity and annealing, the designed primer sequences were checked using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

*hsp*3 gene expression analysis

The *hsp*3 gene expression was analyzed at the transcriptional level; hence, total RNA extraction was carried out. Total RNA was isolated from *L. plantarum* SU-KC1a culture grown under different temperatures. The overnight cultures of *L. plantarum* SU-KC1a (1%) were placed in sterile MRS broth and incubated at 37℃ and 42℃ for one hour. The Patho Gene-spin™ DNA/RNA Extraction Kit (iNtRON Biotechnology, South Korea) was used to obtain total RNA. The RNA quality and concentration were estimated spectrophotometrically. Total RNA was reverse transcribed using the Qiagen OneStep RT-PCR Kit (Qiagen, German) following the manual's procedures. The PCR reaction was carried out using a specific primer targeting the *hsp*3 gene: F (5'-

TGTCAACATCAACGTCGGGTAA-3') and R (5'-CGTAGTCATGGGTCGGTCCT-3'). The reaction was cycled for 30 min at 50°C for the reverse transcription reaction, followed by 15 min at 95°C for initial PCR activation and 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C (denaturation, annealing, and extension phase) and finished by final extension for 10 min at 72°C. PCR amplicons were visualized on 2.5% agarose gel using ethidium bromide (1µg/mL). The intensity of the bands was observed qualitatively to compare the *hsp*3 gene expression between different conditions. PCR amplicons were sent to Apical Scientific (Selangor, Malaysia) for sequencing to confirm the *hsp*3 gene sequence of the obtained band. Sequence similarity results were analyzed with the Basic Local Alignment Search Tool (BLAST). To authenticate the identity of the detected band as the *hsp3* gene, it was excised from the gel and subjected to sequencing. The resultant sequence served as a query in a BLAST alignment against the *hsp3* gene from the WCFS1 strain.

Comparative analysis of stress-related genes in SU-KC1a

The stress-related genes were analyzed by comparing *L. plantarum* SU-KC1a with other *L. plantarum* strains (F75, WCFS1, and SK151). The complete genome sequences of SU-KC1a and F75 were procured from Universitas Pelita Harapan, whereas those of WCFS1 (accession number AL935263.2) and SK151 (accession number CP030105.1) were sourced from the NCBI GenBank. The genome annotations for all four *L. plantarum* strains were conducted following the methodology outlined by Sunardi et al. (2023).

Statistical analysis

Data was reported as mean ± standard deviation (n=5) and analyzed using SPSS (version 26.0, IBM). The Mann-Whitney U tests were used to determine statistical significance. The p-value <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Heat shock tolerance

The ability of *L. plantarum* SU-KC1a to grow under heat-stress conditions is shown in Table 1. The initial cell concentration was 1.58±0.43 (10^6 CFU/mL), while after one hour of incubation at optimal temperature (37°C), the cell number doubled to 3.57±0.11 (10^6 CFU/mL). A modest increase in total cell count was also seen on the culture incubated at 42°C 1.93±0.49 (10^6 CFU/mL). Meanwhile, the cell concentration of the culture incubated at 47°C was slightly decreased to 1.38±0.47 (10^6 CFU/mL). Hence, *L. plantarum* SU-KC1a demonstrated a degree of heat tolerance, maintaining or slightly increasing its cell count under moderate heat stress (42°C), but showing a slight decrease in cell concentration under more severe heat stress (47°C), suggesting its potential limitations in higher temperature environments. Therefore, 42°C was considered the maximum temperature for the growth of *L. plantarum* SU-KC1a.

Growth under sub-lethal conditions

Further experiments were aimed to observe the growth of *L. plantarum* SU-KC1a at 37°C, 40°C, and 42°C. All conditions were started with the same cell concentration. After three hours of incubation, the number of cells from all conditions increased. The culture incubated at 37°C showed the highest growth rate, while the slowest growth rate was at 42°C (Figure 1). Based on these results, the optimal and maximum temperatures for the growth of *L. plantarum* SU-KC1a were 37°C and 42°C, respectively.

Gene expression analysis

Although the whole genome sequence of *L. plantarum* SU-KC1a showed that the strain has *hsp*3 gene (data not shown), the expression of sHSP has not been confirmed. Therefore, total RNA extraction was carried out to evaluate the expression of *hsp3* gene. In normal conditions, *hsp*3 gene expression was observed at the basal level and can be detected; however, the expression increased when the cells were exposed to stress conditions. Total RNA was extracted from *L. plantarum* SU-KC1a grown at optimal (37°C), high (40°C), and maximum temperature (42°C). The extracted RNAs were then reverse-transcribed into cDNA and amplified using a primer targeted to *hsp*3 gene. The obtained amplicons were visualized on agarose 2.5% and analyzed using Gel Analyzer (data not shown). The size of the cDNA obtained from each treatment was about 230 bp (Figure 2).

Table 1. The growth of *Lactiplantibacillus plantarum* SU-KC1a after heat treatment for one hour

Temperature	Cell Concentration (x 10^6 CFU/mL)	
	Initial	Final
37°C	1.58 ± 0.43	3.57 ± 0.11*
42°C	1.58 ± 0.43	1.93 ± 0.49
47°C	1.58 ± 0.43	1.38 ± 0.47
52°C	1.58 ± 0.43	0.34 ± 0.10*

Notes: Data is presented as mean ± SD (n=5); an asterisk (*) in final cell concentration indicates statistically significant differences (p< 0.05) as compared to initial cell concentration

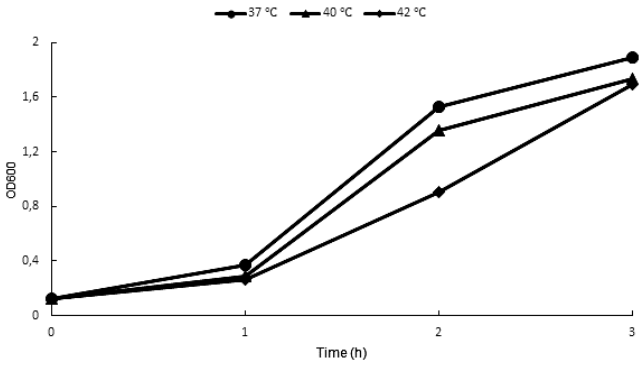

Figure 1. The growth of *Lactiplantibacillus plantarum* SU-KC1a under different temperatures

To confirm that the obtained band was *hsp3* gene, it was extracted from the gel and sequenced. The resulting sequence was then used as a query in a BLAST alignment with the *hsp3* gene from WCFS1 as the subject sequence (Figure 3). The identintities percentage (100%) implies that a partial *hsp3* gene was successfully amplified from *L. plantarum* SU-KC1a.

Comparative analysis

This study assessed the presence of heat stress-related genes in four different *L. plantarum* strains (WCFS1, SU-KC1a, F75, and SK151) using comparative genomics method. Table 2 shows that genes related to stress response in. *L. plantarum* fall into three main categories: protease, molecular chaperone, chaperone-protease, and transcriptional regulator. The chaperone system assists refolding of misfolded proteins, protects functional proteins, and prevents protein aggregation. At the same time, protease is an enzyme that catalyzes proteolysis and degrades irreversibly damaged protein as the last line of defense system. Among the genes encoding protein molecular chaperones, *grpE*, *cL*, *hslO*, *hsp1*, *hsp2*, *hsp3*, *hsp33*, *groES*, *groEL*, *dnaJ*, and *dnaK* were identified. Notably, while *L. plantarum* strains WCFS1 and SK151 possessed the *hslO* gene, SU-KC1a and F75 lacked this gene. Similarly, WCFS1 and SK151 did not have the *hsp33* gene, whereas SU-KC1a and F75 did. This discrepancy arises from naming preferences; both the *hslO* and *hsp33* genes refer to the same gene, indicating that *hslO* is an alternative symbol for the *hsp33* gene.

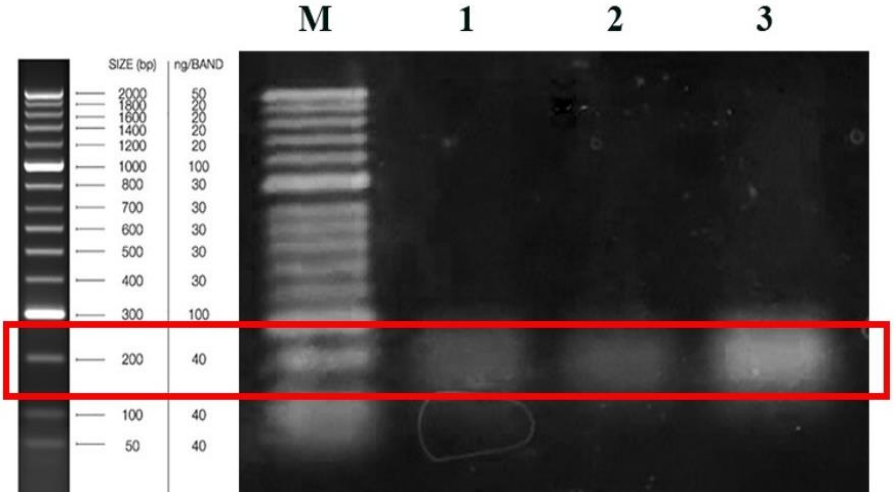

Figure 2. Visualization of *hsp*3 gene expression in *Lactiplantibacillus plantarum* strain SUKC-1a. Notes: 1=37°C; 2=40°C; 3=42°C; M=50 bp molecular ladder (Hyperladder™)

Sequence ID: Query_3771903 Length: 444 Number of Matches: 1

Range 1: 131 to 337 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
383 bits(207)	3e-111	207/207(100%)	0/207(0%)	Plus/Plus

```
Query  1    CGACCAGTTTGACGTTCACTCGCGATAATATTACCATCTTTATCGCTTTCATCACTAATA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  CGACCAGTTTGACGTTCACTCGCGATAATATTACCATCTTTATCGCTTTCATCACTAATA  190

Query  61   CTATCGCGTTTTACAGCAATCGATAATGTGCCATCCCGATACTTCAAGGCAATATTCTGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  CTATCGCGTTTTACAGCAATCGATAATGTGCCATCCCGATACTTCAAGGCAATATTCTGT  250

Query  121  TTATCGATTCCAGGAATATCGACTTTCATCGTGTACTGATCGTCATTTTCTTTAATATCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  TTATCGATTCCAGGAATATCGACTTTCATCGTGTACTGATCGTCATTTTCTTTAATATCA  310

Query  181  GTCTTTAGGACCGACCCATGACTACCA  207
            |||||||||||||||||||||||||||
Sbjct  311  GTCTTTAGGACCGACCCATGACTACCA  337
```

Figure 3. Alignment of *hsp3* gene. Notes: The query sequence refers to the amplified *hsp3* gene obtained from *L. plantarum* SU-KC1a, while the subject sequence pertains to the *hsp3* gene sourced from *L. plantarum* WCFS1. These sequences are aligned using the Basic Local Alignment Search Tool (BLAST)

Table 2. The presence of heat stress response genes in four different *Lactiplantibacillus plantarum* strain

Category	Gene	Annotation	Existence			
			WCFS1	SU-KC1a	F75	SK151
Protease	*ClpX*	ATP-dependent *Clp* protease ATP binding subunit *Clp*X	+	+	+	+
	ClpP	ATP-dependent *Clp* protease proteolytic subunit	+	+	+	+
	hslV	*HslU--HslV* peptidase proteolytic subunit	+	+	+	+
	hslU	ATP-dependent protease ATPase subunit *HslU*	+	+	+	+
	htpX	Zinc metalloprotease *HtpX*	+	+	+	+
Molecular-chaperone	*grpE*	Nucleotide exchange factor *GrpE*	+	+	+	+
	CL.	ATP-dependent chaperone *CL.*	+	+	+	+
	ClpC	ATP binding subunit *ClpC*	-	+	+	-
	hslO	Hsp33 family molecular chaperone *HslO*	+	-	-	+
	(Hsp20) *hsp1*	Hsp20 / *alpha-crystallin* family protein	+	+	+	+
	(Hsp20) *hsp2*	Hsp20 / *alpha-crystallin* family protein	+	+	+	+
	(Hsp20) *hsp3*	Hsp20 / *alpha-crystallin* family protein	+	+	+	+
	hsp33	Molecular chaperone Hsp33	-	+	+	-
	groES	Co-chaperone GroEs	+	+	+	+
	groEL	Chaperonin GroEl	+	+	+	+
	dnaJ	Molecular chaperone DnaJ	+	+	+	+
	dnaK	Molecular chaperone DnaK	+	+	+	+
Chaperone-protease	*FtsH*	ARP-dependent zinc metalloprotease FtsH	+	+	+	+
Transcriptional regulator	*hrcA*	Heat-inducible transcription repressor	+	+	+	+
	CtsR	CtsR family transcriptional regulator	+	+	+	+

Discussion

The results suggested that SU-KC1a exhibited a reduced growth rate as temperatures increased, with 42°C being the upper limit conducive to growth. According to Matejcekova et al. (2016), the maximum temperature of *L. plantarum* was observed at 41.1°C, and the growth rate was 62% slower than at 37°C to reach the stationer phase. In addition, Liao et al. (2010) reported that *L. plantarum* started to decrease at 49°C and kept decreasing drastically at 52°C, 55°C and 58°C. Furthermore, reversible protein damage occurred at 47°C, but cells could still refold the protein, which a group of chaperones such as *dnaK, dnaJ, GroES, GroEL,* and sHSP facilitates. Meanwhile, the decrease of viable cells at 52°C was significant due to rapid and considerable amount of protein damage in a short time, causing irreversible damage and cell death. Therefore, 52°C was lethal for *L. plantarum* (Liao et al. 2010; Arena et al. 2019). The slower growth of *L. plantarum* SU-KC1a at higher temperatures indicated that the cells experienced a disturbance during the growth, which might be caused by structural changes in protein, leading to the inhibition of the enzymatic activities inside the cells. Protein damage will activate various cell repair mechanisms to re-fold the protein and prevent death. According to Adamberg et al. (2003), cell repair mechanisms will allocate the cell's energy for functional systems and repairing damages instead of using energy for replication; this phenomenon is called maintenance energy. Therefore, the growth at sub-optimal conditions will also reduce the growth yield, which is the total mass of cells formed per mass of substrate consumed.

Heat Shock Proteins (HSPs) function as chaperones, aiding in protein refolding during heat stress to prevent denaturation. The expression of heat shock proteins is triggered by the accumulation of damaged proteins in the cytoplasm (Hu et al. 2020). Among the genes associated with heat stress response are *groES, groEL, dnaK,* and *dnaJ*, which can be induced by various stressors and are typically expressed at low levels under normal conditions. In contrast, small heat shock proteins (sHSPs) are particularly relevant for heat stress protection. They are encoded by three genes, namely *hsp1*, *hsp2*, and *hsp3* (Arena 2019). Spano et al. (2005) found that the size of the *hsp3* gene fragment in *L. plantarum* isolated from wine was approximately 450 bp, while the total fragment sequence of the *hsp3* gene in *L. plantarum* WCFS1 is 444 bp (GenBank: AL935263.2). In our study, the fragment length of *hsp3* gene was approximately 247 bp (Figure 2), likely influenced by the different primers used in each investigation. Figure 2 also shows that the fragment from incubation at 42°C appeared clearer than the fragment from incubation at normal temperature 37°C and 40°C, indicating that the increased expression of *hsp3* gene could be induced by heat stress. Theoretically, conventional RT-PCR doubled the template exponentially, thus the difference in relative amount between samples could be measured by the fragment intensity on gel agarose. In other words, the gel-based visualization might be used to determine genes qualitatively. However, RT-qPCR must be used to determine the gene expression quantitatively (Spano et al. 2005; Remans et al. 2014). The gene expression related to heat stress influences the growth and survival of a culture in less ideal environments. Under the heat stress conditions, the cell's energy was allocated to synthesize heat stress genes such as *hsp3* to repair cell damage. Hence, the energy for replication declined, and the growth rate decreased. Energy allocation allows bacteria to survive when imposed in stressful environments (Adamberg et al. 2003; Arena et al. 2019). This might explain why the cultures incubated at higher temperatures experienced relatively slower growth, but the fragment of *hsp3* gene appeared more noticeable after gel visualization.

The role of *hsp1* is more relevant to low-temperature survival in controlling and improving membrane fluidity to support its cryoprotective function, while the *hsp3* gene is more responsible for heat stress protection. On the other hand, *hsp2* gene does not exhibit relevancy to heat or low-temperature stress. Capozzi et al. (2011) state that the *hsp2* gene regulates membrane fluidity, and the gene's loss did not impact resistance to heat or other stress (Papadimitriou et al. 2016; Arena et al. 2019). In regulating the stress genes, *L. plantarum* possessed two main regulators to control genes related to heat stress response: *hrcA* as regulator Class I and *ctsR* as regulator Class III. A regulator is a gene that controls one or more genes by producing a repressor protein to determine gene expression upon the presence or absence of a repressor protein in the operon. Both regulators in *L. plantarum* act as a repressor that will suppress gene expression. Regulator *hrcA* regulates expression of *groEL*, *groES*, *grpE*, *hsp1*, *hsp2*, *hsp3*, *dnaK*, and *dnaJ*, while *ctsR* encodes *clpP*, *clpX*, *clpC*, *hsp1*, *hsp2*, *hsp3*, *grpE*, *dnaK*, *hslU*, and *hslV* gene; both regulators can regulate several genes at the same time (Van Bokhorst-Van de Veen et al. 2013; Chiu et al. 2015; Papadimitriou et al. 2016).

Generally, there are no significant differences in heat shock-related genes in the four *L. plantarum* strains because the genes are mostly conserved and essential for survival. Stress-related genes are also expressed constitutively during normal conditions to assist protein folding and repair misfolded proteins that may still occur.

ACKNOWLEDGEMENTS

This research was supported by the Research and Community Service Centre (LPPM), Universitas Pelita Harapan, Tangerang, Indonesia (grant number P-02-FaST/VIII/2022). The authors would like to thank the Department of Biology, Universitas Pelita Harapan for providing facilities at the Basic (203) and Advanced (407) Biology Laboratories.

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