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Cold Stress Response Genes of *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-7 Support the Ability to Survive in Low-Temperature Conditions

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ABSTRACT

Probiotics are widely consumed in various food matrices to provide health benefits to the host. The viability of probiotic cells is influenced by several factors, including exposure to high temperatures during the production process and low temperatures during storage. In this study, we report the response to cold stress of *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and Mut-7 after 24 h of storage at 4°C and -20°C. The cell number of *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and Mut-7 in low-temperature condition had no significant differences than their initial number: 11.88 log CFU/ml and 11.62 log CFU/ml at 4°C; 11.51 log CFU/ml and 11.47 log CFU/ml at -20°C for Mut-3 and Mut-7 respectively. The results indicated the survival capacity of *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and Mut-7 at low temperatures. The genes encoding cold shock proteins for the response to cold stress were evaluated by genome sequencing. The *CspA/CspC* genes of *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and Mut-7 possibly play a role in maintaining cell resistance at low temperatures, since the genes products predicted to have conserved motifs in the RNA binding protein (RNP) -1 and RNP-2 responsible for cold response stress which are similar to those in other bacteria.

1. Introduction

Probiotics are living microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO 2002). They have been applied in several food matrices, such as; ice cream, fermented milk products, cheese, chocolate, crackers, breakfast cereal, chips, peanut butter, and crispy granola (Forssten *et al.* 2011). The microorganisms generally belong to the genera of *Lactobacillus* and *Bifidobacterium*; also, species from other genera (Forssten *et al.* 2011). Probiotics used in food products should maintain their viability under various temperature conditions and must survive in sufficient quantities until the end of shelf life of the food of the food (Fenster 2019).

Organisms are exposed to low temperatures during frozen storage and low temperature fermentation or storage of fermented products (Wouters *et al.* 2000). Low temperatures are usually used to keep starter cultures or probiotics in frozen or freeze-dried form with the addition of cryoprotectants to protect the cell from damage due to freezing (Santivarangkna *et al.* 2011).

The cold shock on the cells can reduce the efficiency of translation, transcription, and DNA replication because of the stabilization of RNA and DNA secondary structure (Graumann and Marahiel 1998). A set of proteins, called cold shock protein (Csp), is induced when cells are exposed to low temperature (Papadimitriou *et al.* 2016). Cold shock proteins (Csps) are acting as RNA chaperons that help RNA trapped in an unproductive folded state. Such molecular functions allow Csps to participate in the regulation of nearly all gene expression steps involving RNA, including transcription, translation, and RNA turnover (Mihailovich *et al.* 2010). Csps can reduce the increased amount of secondary RNA folding when cells are exposed to low temperatures

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(Wouters *et al.* 2000). Csps are also involved in cell adaptation to low temperatures, cell growth, nutrient stress, and stationary phase (Graumann and Marahiel 1998). Csps have a minor molecular weight of approximately 7 kDa (Wouters *et al.* 2000).

Studies on Csps in bacteria indicated that CspA is the major cold-shock protein of *Escherichia coli*, and several others cold shock proteins belong to the CspA family (Jiang *et al.* 1997). It has nine homologous proteins, CspA to CspI but, not all of them are induced by cold shock (Wang *et al.* 1999). Cold-inducible protein in *E. coli*: CspA, CspB and CspG are regulated differently (Bae *et al.* 1997). *Bacillus subtilis* has CspB, CspC, and CspD, which increased mRNA levels of the cold shock genes and the most dominant protein spots according to the 2D PAGE approach after temperature downshifts from 37°C to 18°C were cold-shock protein CspB and CspD (Kaan *et al.* 2002). In psychrophilic *Listeria monocytogenes*, CspA and CspD upregulated at 4°C (Hingston *et al.* 2017).

Lactic acid bacteria also have several cold-shock inducible proteins (Derzelle *et al.* 2003; Mayo *et al.* 1997; Song *et al.* 2014; Sauvageot *et al.* 2004). *L. plantarum* being exposed by downshifting temperature from 37°C to 10°C resulted in the high level of cspL and cspP mRNAs observed after one hour at 10°C (Mayo *et al.* 1997). Song *et al.* (2014) reported that after applying adapted cold stress to *L. plantarum* L67 for six hours, cspP and cspL were upregulated and the cells maintain their viability after four cycles of freeze-thaw challenge. Other studies found that *L. plantarum* UL497 has CspL, CspC, and CspP and can survive after cold shock from 30°C to 8°C in the growing log phase after five hours (Nguyen and Vo 2018).

Our laboratory isolated indigenous LAB from fermented cassava-based products, *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-3 and *Lantiplantibacillus plantarum* subsp. *plantarum* Mut-7 (previously known as *Lactobacillus plantarum* Mut-3 and *L. plantarum* Mut-7, respectively) and had tested their capacities as probiotics (Rahayu *et al.* 2016). According to Harahap *et al.* (2021), *L. plantarun* Mut-7 had the potential to reduce the total of *Escherichia coli* and Coliform non-*E. coli* in several subjects through ingestion of milk containing *L. plantarum* subsp. *plantarum* Mut-7; increased the total LAB and *L. plantarum* subsp. *plantarum* in subjects' fecal matters. However, the information regarding cold stress response in those strains is limited. Since the preparation of probiotics cells may include low temperature treatments such as refrigeration and freezing, the studies of cell viability after exposed to the cold stress and their corresponding genes are necessary to expand future application of *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7 as indigenous probiotics.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

Lactiplantibacillus plantarum subsp. *plantarum* Mut-3 and *Lactiplantibacillus plantarum* subsp. *plantarum* Mut-7 (previously known as *Lactobacillus plantarum* Mut-3 and *Lactobacillus plantarum* Mut-7 respectively) cultures were obtained from Food Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia. For analysis, we cultured and propagated the bacteria in De Man Rogosa Sharpe (MRS) (Merck, Darmstadt, Germany) at 37°C for 24 h. The cultures were stored at 5°C and recultured every once a week in MRS broth.

2.2. Cold Shock Treatment

Stock cultures were grown in 250 ml of fresh MRS broth followed by incubation at 37°C, 24 h. Cells of bacteria were harvested by centrifugation (Heraeus Sepatech, Germany) at 3,500 rpm for 10 min and the remaining pellet were resuspended in a sterile solution of 0.85% NaCl divided into three groups. The first group was maintained as a control to obtain the initial number of cells, second and third were treated overnight at 4°C and -20°C, respectively. This experiment was performed four times.

2.3. Enumeration of Cells

Serial dilutions of cell suspensions were performed before and after low-temperature treatment. A volume of 1 ml of each dilution was then poured into three plates and MRS Agar was added with $CaCO_3$ 0.5%, followed by incubation at 37°C for 24 hours. The cell colonies which grew were counted.

2.4. Genome Sequencing and *In Silico* Analysis of Cold Shock Proteins

Genome sequencing of *L. plantarum* subsp. *plantarum* Mut-7 and Mut-3 was performed using the Illumina NovaSeq 6000 Sequencing Platform followed by genome assembly using methods described elsewhere (Suroto *et al.* 2021). Cold stress response proteins were identified by Rapid Annotation in Subsystem Technology (RAST) V.20 SEED (http://rast.nmpdr.org) (Overbeek *et al.* 2014). The proteins were compared to other cold stress response proteins in the Uniprot data base using Protein Blast by the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All proteins were further

aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (https://www.ebi.ac.uk/Tools/msa/muscle/).

2.5. Statistical Analysis

The analysis was carried out from replication of the experiment replication of the and replication of the and replication of the and twice and replication of the analysis twice to obtain the mean value ± standard deviation (SD). The results of the data were processed using one-way ANOVA and continued with Duncan's multiple range test to determine the difference between treatments with a significance level (P<0.05).

3. Results

3.1. Cold Shock Treatment

The initial numbers of *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7 were 11.78 log CFU/ml and 11.56 Log CFU/ml. Both strains showed no significant differences between initial numbers, after storage at low temperature (4°C), and after storage at freezing temperature (-20°C) (Table 1). The phenotypic results shown by the cell viability test of *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7 indicated that cells might survive in low-temperature conditions and freezing temperatures.

Table 1. Viability of *Lactiplantibacillus plantarum* subsp. *plantarum* after storage at low and freezing temperature for 24 hours

Strains	Log CFU/ml		
	Initial number	4°C	-20°C
Mut-3	11.78±0.60[a]	11.88±0.19[a]	11.51±0.44[a]
Mut-7	11.56±0.53[a]	11.62±0.43[a]	11.47±0.33[a]

Means with the same superscripts indicate no significant difference (P<0.05)

3.2. Detection of Cold Shock Protein

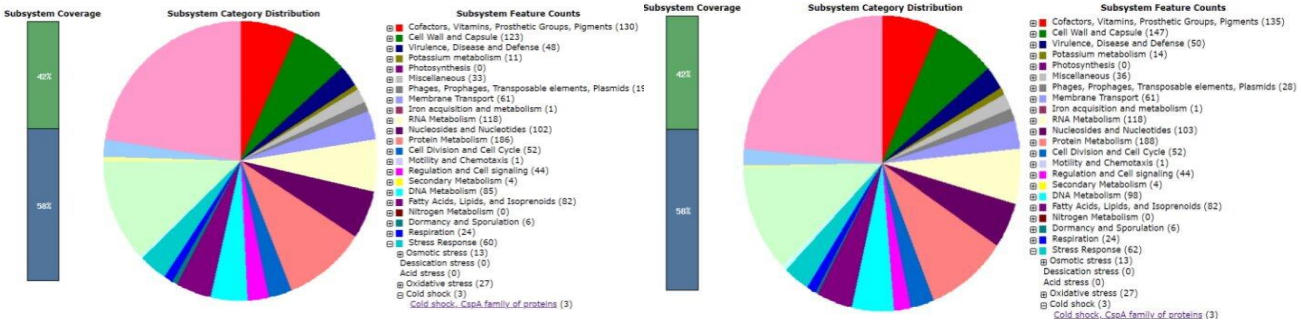
The information available on RAST subsystem is shown in Figure 1. *L. plantarum* subsp. *plantarum* Mut-3 contains 60 genes from all stress response categories, while *L. plantarum* subsp. *plantarum* Mut-7 has 62 genes.

RAST detected three genes in the stress response category and cold shock subcategory, they encoded two cold shock protein: CspA and CspC. There are two copies of CspA and one copy of CspC in Mut-3 and Mut-7, respectively (Table 2).

Two CspAs were found in each Mut -3 or Mut-7 and one of the CspA in Mut-3 and Mut-3 showed exactly identical cold shock proteins to those in *L. plantarum* WCSFI. Furthermore, the CspCs of Mut-3 and Mut-7 showed high identity with CspLA of *Listeria monocytogenes* EGD-e. Interestingly, CspA from Mut-3 and Mut-7 also showed high identity 74.60 -76.56% to CspLA (data not shown).

Since CspA and CspC from Mut-3 and Mut-7 have a high identity of the amino acid sequence, we performed multiple alignment of Csps from Mut-3 and Mut-7 with other well-studied cold shock proteins in lactic acid bacteria and psychrophilic bacteria *Listeria monocytogenes* EGD-e to obtain more information on the conserved motif they might share (Figure 2).

As shown, two RNA Binding motifs (RNP1 and RNP2) are observed in CspAs and CspCs of both *L. plantarum* subsp. *plantarum.* But, in the RNP1 of CspAs showed different amino acid sequence, one of CspA in Mut-3 and Mut-7 have tyrosine at third position while the other CspA have phenylalanine instead. The CspCs protein both in Mut-3 and Mut-7 have phenylalanine at third position of RNA binding motif. The second RNP of all CspAs and CspCs showed the same sequence of amino acids.



A B

Figure 1. (A) Subsystem distribution of *Lactiplantibacillus plantarum* subsp. *plantarum* Mut-3 on RAST, (B) subsystem distribution of *Lactiplantibacillus plantarum* subsp. *plantarum* Mut-7 on RAST

Table 2. Deduced function of the cold shock family proteins

Microorganism	Gene	Size[a]	Homolog[b] and Origin	Identity/Similarity (%)	Proposed function
L. plantarum subsp. *plantarum* Mut-3	*cspA*	66	cspP (P71478.1) *Lactiplantibacillus plantarum* WCFS1	100/100	Cold-shock protein
	cspA	66	cspL (P96349.1) *Lactiplantibacillus plantarum* WCFS1	100/100	Cold-shock protein
	cspC	66	cspLA (P0A355.1) *Listeria monocytogenes* EGD-e	85/86	Cold-shock protein
L. plantarum subsp. *plantarum* Mut-7	*cspA*	66	cspP (P71478.1) *Lactiplantibacillus plantarum* WCFS1	100/100	Cold-shock protein
	cspA	66	cspL (P96349.1) *Lactiplantibacillus plantarum* WCFS1	100/100	Cold-shock protein
	cspC	66	cspLA (P0A355.1) *Listeria monocytogenes* EGD-e	85/86	Cold-shock protein

[a]Numbers refer to amino acid residues
[b]Parenthetical codes are accession numbers from the National Center for Biotechnology Information

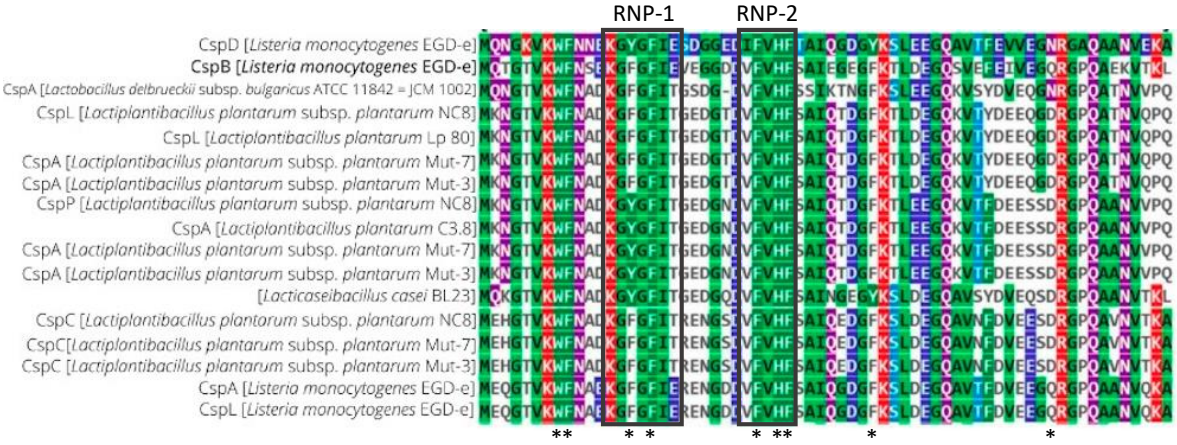


Figure 2. Multiple alignments of CspA/CspL amino acid sequences using MUSCLE RNA Binding Motifs are indicated by black boxes, while important residues for nucleic acid binding are indicated by asterisk

4. Discussion

When microbial cells are subjected to low-temperature conditions, cells can be injured by formation of intra- and extracellular ice crystals and the disassociation of cellular lipoproteins due to water loss in the frozen state (El-Kest and Marth 1992). Refrigeration and freezing treatment on Mut-3 and Mut-7 for 24 hours did not change the viability of those two *L. plantarum* subsp. *plantarum*. Our results were in contrast to the studies in *L. plantarum* L67 which showed 20% decrease in cell survival after freezing at -70°C for 24 the (Song *et al.* 2014) and *L. plantarum* UI497 which showed 80% decrease in cell survival after storage at -20C° for 24 hours (Nguyen and Vo 2018).

Proteins that play a role during cells exposure to low temperature of freezing temperature are cold shock proteins (Csps). Csps are small (7 kDa) proteins that act as RNA chaperones to help mRNA folding and protein synthesis at low or freezing temperatures (Wouters *et al.* 2000). The cold shock protein is a nucleic acid chaperone that binds to RNA and DNA to control processes such as replication, transcription, and translation in bacterial cells.

According to Nguyen and Vo (2018), in *L. plantarum* UL497 there are two conserved RNA binding proteins (RNP), namely K-G-F-G-I-T (RNP-1) and V-F-V-H-F (RNP-2), those sequences are similar to those of CspA of Mut-3 and Mut-7. These binding motifs are conserved in other bacteria with slight modification in the hydrophobic amino acid for nucleic acid binding (Newkirk *et al.* 1994; Schmid *et al.* 2009; Sauvageot *et al.* 2006; Yu *et al.* 2019). The modification of binding motif also found in RNP1 of CspA in Mut -3 and Mut-7 especially at third residues but not in CspCs. Little amino acid differences in RNP might contribute to the specificity and affinity of Csp for specific RNA or DNA

regions, which could be responsible for specialization or differences in the role of Csp (Muchaamba *et al.* 2021). According to the research of Schmid *et al.* (2009) in *Listeria monocytogenes* EGD-e treated at 4°C, *cspA* was regulated so that cell growth at low temperatures could occur, and from among *cspA, cspB,* and *cspD*, only *cspA* showed the highest expression level indicated that it is necessary for cell growth at low temperatures. The up-regulation of *cspA* in *Listeria monocytogenes* Lm1 was also observed when cells are treated and observed at 4°C (Hingston *et al.* 2017).

Research on the cold shock protein is often performed using *Escherichia coli*. One of the Csp owned by *E. coli* is CspA with RNP-1: K-G-F-G-F-I-T-P and RNP-2: V-F-V-H-F. In *E. coli*, *CspA* has a set of aromatic amino acids tryptophan (W), phenylalanine (F), and tyrosine (Y), where these amino acids are needed for DNA or RNA binding. A set of hydrophobic amino acid residues was also obtained, namely vacillin (V), to form a hydrophobic protein core (Newkirk *et al.* 1994). The amino acid sequences of *CspA* and *CspC* belonging to *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7 showed similar motif to that in *E. coli* and also have aromatic amino acids, W8, F9, F15/Y15, F17, F27 (the number is the order in which the amino acid residues are located). Additionally, both have hydrophobic amino acid residues I18 and V26. However, *L. plantarum* subsp. *plantarum* Mut-7 and Mut-3 have shorter *Csps* compared to those in *E. coli*.

According to the results of research and literature study, it was found that the *cspC* gene encoding the CspC protein possessed by *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7 had similarities in conserved motifs with *Listeria monocytogenes* EGD-e, which regulates CspA when cells were at 4°C. *L. plantarum* subsp. *plantarum* Csp may be active when cells are given a reduction in temperature resulting in increased cell survival. Thus, especially the CspC found in *L. plantarum* subsp. *plantarum* Mut-3 and Mut-7, may also perform its function to protect cells from cold threats and be active at low temperatures.

In conclusion, genome sequencing data showed that *Lactiplantibacillus plantarum* subsp. *plantarum* Mut-3 and Mut-7 have *cspA* and *cspC* genes that produce CspA and CspC proteins. Those protein may be responsible for cells to survive in low temperature environments through comparisons of conserved protein motifs of CspA/CspL from psychrophilic *Listeria monocytogenes* EGD-e and Csps in other *L. plantarum* strains. This result was supported by cell viability data at 4°C and 20°C, which showed that cells can maintain their viability at these temperatures.

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