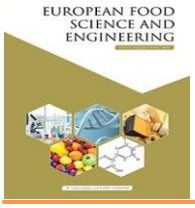

European Food Science and Engineering

Eur Food Sci Eng 2024, 5 (2), 96-100
doi: 10.55147/efse.1559159
https://dergipark.org.tr/tr/pub/efse

Dynamic expression of heat-shock and acid-tolerance related genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* LBB.B5 in milk

Miroslava Terzieva-Nakeva* and Zoltan Urshev

R&D Department, LB Bulgaricum PLC, Sofia, Bulgaria

ARTICLE INFO

Research Article
Article History:
Received: 1 October 2024
Accepted: 15 November 2024
Available Online: 30 December 2024
Keywords:
Acid-tolerance
Gene expression
Heat-shock
Lactobacillus bulgaricus

ABSTRACT

The present study monitors the dynamic expression of heat/cold-shock related genes (*hsp60* and *cspA*) and genes putatively contributing to acid-tolerance (*ornB*, encoding ornithine decarboxylase and *thrB* and *thrC*, involved in threonine synthesis) in *Lactobacillus delbrueckii* ssp. *bulgaricus* LBB.B5. Expression levels were monitored by RT-qPCR for 7 hours of fermentation at 42 °C and then until the 24th hour under cold storage. Two distinct patterns in the expression dynamics were observed. Genes *cspA*, *ornB*, *thrB* and *thrC* had maximal expression at 5h of the fermentation with levels of 11.6, 6.8, 3.9 and 2.4 times the control (3h), respectively, coinciding with the transition of the culture from exponential to stationary phase at a pH threshold of 5.0. Gene *hsp60* showed a different pattern with gradually increasing expression throughout the fermentation process and cold storage reaching 6.4 times the control. The upregulation of threonine and cold-shock protein synthesis with the onset of the stationary phase may suggest that like ornithine decarboxylase, they go beyond amino-acid anabolism or managing cold stress, but rather facilitate the transition of the cells to stationary phase and/or to acidic conditions. The gradual upregulation of *hsp60* may reflect cell adaptation to growth at 42 °C and cold storage.

1. Introduction

Today, direct vat starters (DVS) are becoming the standard in dairy production (Mullan, 2006) with the process of starter preparation including a freeze-drying step (Fonseca et al., 2015). However, *Lactobacillus delbrueckii* ssp. *bulgaricus* strains, used extensively in yoghurt starters, are notorious for their poor survival rate during freeze drying with losses in viable cell counts reaching 90% (Rumian et al., 1993). On the other hand, it has been demonstrated that robustness of lactic acid bacterial cultures to drying processes is dependent on strain-specific gene content, transcriptome signatures and expression of particular genes, related to stress, for example heat and oxidative stress (Dijkstra et al., 2014). Therefore, it is essential that the bacterial cells enter the drying process in the most favourable physiological state (Shao et al., 2014).

In the production process different stress factors play a role in the conditioning of the cell, such as heat, cold and acid stress. The response of the cell to these factors can be monitored by the expression of genes that are related to heat/cold shock and acid tolerance. One example is the cold shock proteins, encoded by *cspA* and *cspB* which are highly structurally conserved in lactic acid bacteria (Kim et al., 1998). The mechanism of action of cold shock proteins has been demonstrated to include stabilization of mRNA structures, resulting in post-transcriptional regulation under stress conditions (Zhang et al., 2018). Both *cspA* and *cspB* are well studied in *L. delbr.* ssp. *bulgaricus* and *cspA* has been found to be a temperature-inducible gene (Serror et al., 2003).

In microorganisms heat shock proteins encoded by the *hsp60 (groEL)* and *hsp70 (dnaK)* genes, originally identified by their increased abundance following heat shock, have been characterized as chaperones, responsible for protecting the newly synthesized polypeptides from aggregation and improper folding to a mature protein (Bukau & Howich, 1998). Indeed, increased abundance of GroEL and DnaK following heat shock has been demonstrated in strains of *Lactobacillus acidophilus*, *L. casei* and *L. helveticus* (Broadbent et al., 1997). An increased expression of the *hsp60* gene was also observed in strains of *L. delbr.* ssp. *bulgaricus* when cultivated at elevated temperatures (Shao et al., 2014).

Few systems related to acid tolerance have been suggested for *L. delbr.* ssp. *bulgaricus*, mainly being H+ transporting ATPases and cation:proton antiporters (van de Guchte et al., 2002). Enzymes, such as ornithine decarboxylases may have contributed to acid tolerance in this species (El Kafsi et al., 2014). Others have suggested that the ability of certain strains of *L. delbr.* ssp. *bulgaricus* to synthesize *de-novo* particular amino-acids correlates with their higher acid tolerance (Li et

*Corresponding author
E-mail address: m.terzieva@lbbulgaricum.bg

al., 2020). However, in this species *de-novo* synthesis pathways have remained intact only for 3-6 amino-acids with the striking exception of threonine, whose synthesis has remained conserved in all *L. delbr.* ssp. *bulgaricus* strains (Liu et al., 2012, El Kafsi et al., 2014).

In the present study in order to assess the physiological state of *L. delbr.* ssp. *bulgaricus* cells at different time points in fermentation, we followed the expression of two heat/cold shock-related genes (*hsp60* and *cspA*) and genes putatively contributing to acid-tolerance (*ornB*, *thrB* and *thrC*) in the industrial strain LBB.B5 during a process resembling its large-scale production.

2. Materials and Methods

2.1. Strains and culture conditions

Strain *L. delbr.* ssp. *bulgaricus* LBB.B5 was originally isolated in 1969 in the village of Dalboki, Bulgaria from homemade yogurt and maintained in the culture collection of LB Bulgaricum PLC (Sofia, Bulgaria). Since then, this strain has become the component of the first industrial starters for traditional Bulgarian yoghurt (Kondratenko et al., 1979) with its draft genome assembled in 2016 (Urshev et al., 2016). For the purpose of the study a fresh milk culture of strain LBB.B5 was inoculated at a 3% rate into sterile 10% reconstituted skim milk powder with RNA isolation and measurement of pH values and viable cell numbers at 3, 5 and 7 h of fermentation at 42 °C, followed by cold storage at 4 °C until the 24th hour. Cell counts were evaluated by plating ten-fold dilutions of milk samples to MRS agar plates and anaerobic incubation for 48 h at 37 °C.

2.2. RNA isolation and evaluation of relative expression

RNA was isolated directly from milk samples. Three milliliters of culture were mixed with three volumes of cold 2% sodium citrate and centrifuged at 3000 *xg* and 4 °C for 10 min. The resulting pellet was resuspended in 10 mL of the same solution and centrifuged again. If residual milk was still observed this last step was repeated once more. Finally, the pellet was washed with 1 mL TE buffer, centrifuged at 10000 *xg* for 5 min and resuspended in 0.1 mL TE. All subsequent treatments were performed with the E.Z.N.A. Bacterial RNA Kit (Omega Bio-tek Inc) according to the producer's instructions. The quality of the obtained RNA was assessed spectrophotometrically (OD260/OD280 within the range of 1.8-2.0) and by denaturing RNA electrophoresis (Masek et al. 2005).

For subsequent analysis all RNA preparations were additionally treated with DNAseI (DNase Max® Kit, Quiagen) to remove residual DNA. Consensus sequences for the target genes *cspA*, *hsp60*, *ornB*, *thrB* and *thrC* were obtained based on the genomes of *L. delbr.* ssp. *bulgaricus* strains LBB.B5, 2038 and ATCC BAA-365 and ATCC 11842T (GenBank Acc. Nos. LUGK00000000, CP000156, CP000412 and CR954253). Specific forward and reverse primers were designed with the Primer3Plus software, version 3.3.0 (www.primer3plus.com, accessed on 12.04.2024) with product size set in the range of 100-300 nt and melting temperature of 58-62 °C. All primers, including the primer pair gyrBF/gyrBR for the control housekeeping gene *gyrB* are listed in Table 1.

Reverse transcription PCR amplifications were performed on a CFX Real-Time System (Bio-Rad Laboratories) in 20 microliter PCR reactions, following the iTaqTM Universal SYBR® Green One-Step Kit's protocol (Bio-Rad Laboratories). Each preparation was run in duplicate for 35 cycles with a no-template negative control and no-reverse transcriptase-control. Relative expression was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen, 2001) with samples obtained at 3h of incubation used as a reference control.

3. Results

The cell morphology of *L. delbr.* subsp. *bulgaricus* LBB.B5 could be described as medium to long rods, with volutin granules well-formed already after 3h of incubation and increasing in size and number during subsequent hours of fermentation and cold storage (Figure 1). No difference in cell morphology could be determined between cells grown for 5h and 7 h or after cold storage (24 h).

Viable cell counts changed from 6.88 to 8.40 log (CFU/mL) with maximal cell counts measured at 5 h of incubation, then remaining constant for two more hours of fermentation at 42 °C and during cold storage at 4 °C. Acidity decreased continuously to pH of 4.68 with no further changes during cold storage (Figure 2). Notably, at 5 h of incubation, when the culture reached maximal cell counts, the pH of the medium was 5.0.

Table 1. List of primers used for RT-PCR of target genes

Target	Primer	Sequence (5'-3')	Reference
Cold-shock protein A, *cspA*	cspA-30f	TGCTGATAAGGGCTTTGGGT	
	cspA-173r	TGAGGTCCTCGATTGCCTTG	This study
Heat-shock protein, *hsp60*	hsp60-98f	CCAATTGCACAAGAACAGCCA	
	hsp60-251r	GCGGGAGTCTTCAATGGTGA	This study
Ornithine decarboxylase, *ornB*	orndec2f	ATAGCACCAGCAGGATGACG	
	orndec2r	CGGCTGTTGTTGTGCGTAAA	This study
Homoserine kinase, *thrB*	thrbf	CATGCTCATGGCCGACATTG	
	thrbr	TGCCATGATCGCCTACATCC	This study
Homoserine synthase, *thrC*	thrcf	AGCGAAAACAGCGACAACAC	
	thrcr	CGGCGAAGTAGTAGACGACC	This study
DNA gyrase B, *gyrB*	gyrBF	GGGTCGTTGAAGAGCTGAAGG	
	gyrBR	GTTTCCGCCGTGTCCTTACG	Yungareva & Urshev (2018)

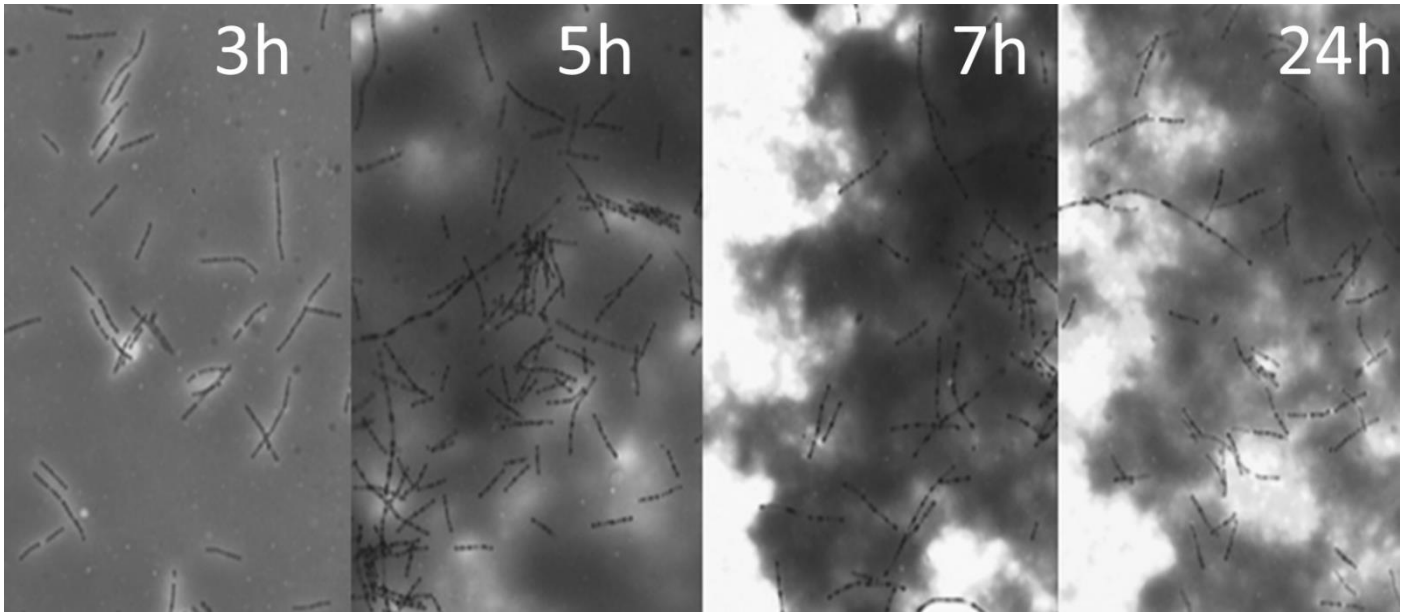

Figure 1. Microscope observation of *L. delbrueckii* ssp. *bulgaricus* LBB.B5 grown in milk at 42 °C for 3 h, 5 h, and 7 h, followed by cold storage until 24 h. Methylene blue staining, 1000 x magnification.

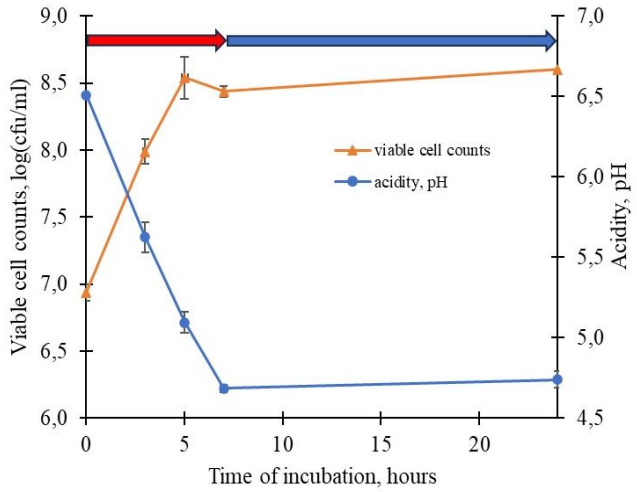

Figure 2. Viable cell counts of *L. delbrueckii* ssp. *bulgaricus* LBB.B5 and changes in pH for 7h incubation at 42 °C followed by cold storage until 24 h. Average values and error bars derived from two independent trials.

The expression dynamics of the analysed genes followed two distinct patterns (Figure 3). The first pattern, that of *cspA*, *ornB*, *thrB* and *thrC*, showed maximal expression levels of 11.6, 6.8, 3.9 and 2.4 times the control, respectively, at 5 h of the fermentation. At this point a transition from exponential to stationary phase was observed with acidification crossing the pH 5.0 threshold (Figure 2). Further into the stationary phase the expression of these four genes decreased, while at the end of the cold storage period it reached levels close to or below the control (3 h).

The second pattern was characteristic for *hsp60* where gradually increasing expression levels were measured, including during cold storage when expression of 6.4 times the control was reached. In full contrast to the other four analysed genes, expression of *hsp60* increased substantially during stationary phase and this trend remained also valid after transferring the culture to cold storage. Of the five tested genes, *cspA* showed the highest dynamic range. Notably, the two independent fermentation trials yielded reproducible results for all tested genes confirming the observed trends in the expression levels (see error bars in Figure 3).

4. Discussion

The expression of genes in the microbial cell depends on culture conditions, but also shows dynamic changes in the course of a batch cultivation/fermentation. For *L. acidophilus* strain it has been demonstrated that 21% of its 1864 open reading frames were expressed differentially in the course of milk fermentation (Azcarate-Peril et al., 2009). A succession in upregulated state was observed for genes related to translation and ribosomal structure (4 h), amino-acid transport and metabolism (8 h) and cell wall and membrane biogenesis (12 h). Therefore, in the present study the expression of the genes of interest was followed throughout the fermentation process with conditions selected to resemble an industrial process.

The genes included in the experiment were selected based on their potential contribution to the adaptation of the bacterial cells to subsequent production steps, such as freeze-drying. At the end of fermentation the bacterial mass is cooled, stored at low temperature for variable period of time and then frozen to continue with the lyophilization process. Therefore cold-shock genes (*csp*) are the obvious target for studying the cell readiness to survive cold storage and freezing. In *L. delbr.* subsp. *bulgaricus* at least two *csp* genes have been described, *cspA* and *cspB*, with increased transcription after shift to low temperature observed only for *cspA* (Serror at al., 2003). Under the selected growth conditions in our study, maximal expression of *cspA* was measured after 5h of incubation at 42 °C, but not after cold storage.

The term “cold shock-protein” (Csp) was initially introduced on the basis of the observed induction of Csp synthesis in *Escherichia coli* after a shift to low temperature (Goldstein et al., 1990), but other studies showed that Csp was also detectable under non-stress conditions and *cspA* mRNA levels were rather dependable on cell density (Brandi et al., 1999). The results from the present study would support the latter observation as expression of *cspA* in *L. delbr.* subsp. *bulgaricus* LBB.B5 reached its maximum together with maximal viable cell counts, i.e. highest cell density.

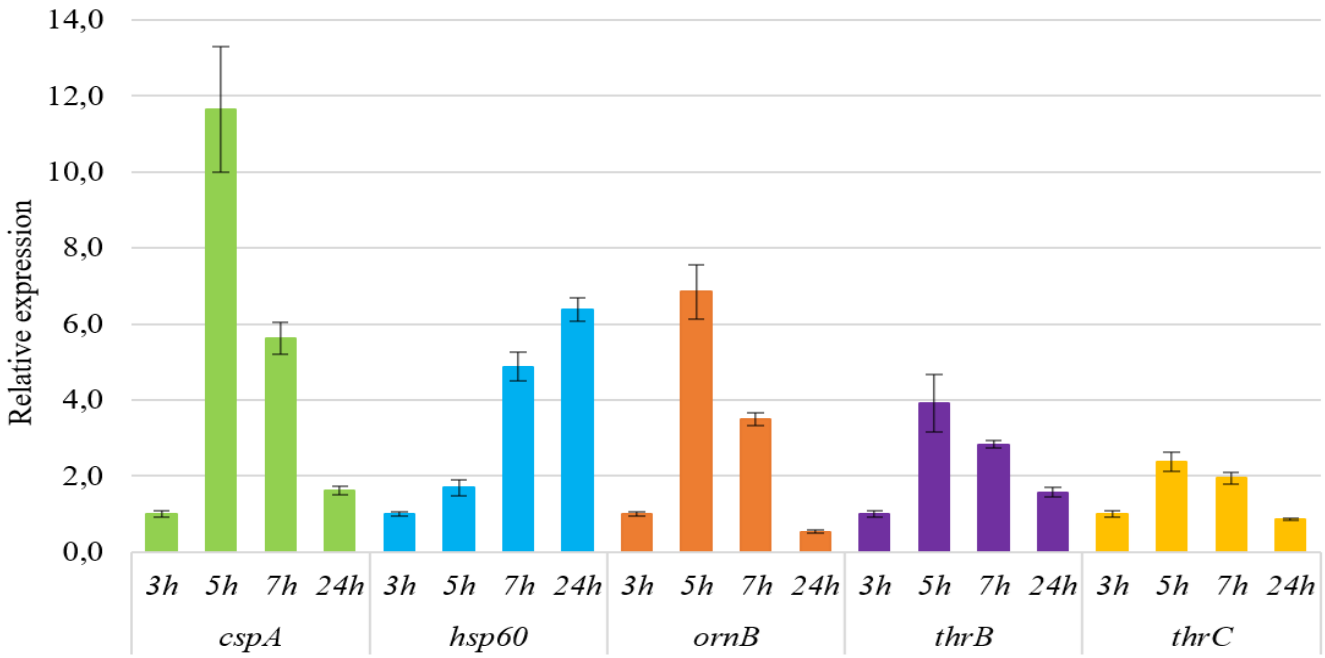

Figure 3. Dynamic expression of heat shock-related genes (*hsp*60 and *csp*A) and genes putatively contributing to acid-tolerance (*orn*B, *thr*B and *thr*C) in *L. delbrueckii* ssp. *bulgaricus* LBB.B5 in milk medium. Relative expression calculated based on control samples at 3 h. Average values and error bars derived from two independent trials.

Moreover, it was found that shift to lower temperatures changes the conformation of *cspA* mRNA increasing the translation rate into Csp, while on the other hand Csp itself can bind to *cspA* mRNA downregulating its own translation (Zhang et al., 2018). Both these mechanisms are posttranscriptional regulation events that are unrelated to the expression of *cspA* at a transcriptional level. Nevertheless, the observed peak in expression levels of *cspA* at 5h of incubation at 42 °C in this study suggests that cells of *L. delbr.* subsp. *bulgaricus* LBB.B5 were in the best physiological state for end of incubation and transfer to cold storage at this time point of the production process. Notably at 5h the pH of the medium was around 5.0.

Heat-shock proteins are chaperones that facilitate correct folding of newly synthesized proteins and as elevated temperatures require more strict control over protein assembly, shift to high temperature results in upregulation of *hsp* genes. However, heat is not the only *hsp* inducer, as increased expression levels in lactobacilli have been reported also after osmotic stress due to elevated salt concentration (Wu et al., 2016). Stress conditions other than heat shock, such as acid stress have been demonstrated to induce heat shock proteins (Lim et al., 2000). Therefore, the induction of *hsp* may be considered as an indication of the onset of stress conditions that influence the folding, assembly and translocation of proteins (Bukau & Horwich, 1998). In the present study *L. delbr.* subsp. *bulgaricus* LBB.B5 was cultured at constant temperature, followed by cold storage, without actually performing a heat-shock step. Nevertheless, the expression of *hsp60* increased constantly with time throughout the experiment. The results from the expression analysis of *hsp60* in our study suggest that the selected temperature (42 °C) in combination with a high inoculation rate (3%) and prolonged incubation (7 h) resulted in increasing the stress burden on the tested strain. Ending the fermentation at 5 h when the maximal number of viable cells was reached may be advantageous for the further processing of the *L. delbr.* subsp. *bulgaricus* LBB.B5 preparation.

The present study aimed to determine the dynamics of *cspA* and *hsp60* expression in the course of fermentation and cold storage. Fermentation temperature of 42 °C and cold storage at 4 °C were selected to keep the conditions close to the industrial production process. However, it should be noted that temperatures different form the selected ones, may result in different expression levels of these two genes. For four *L. delbr.* subsp. *bulgaricus* strains Shao et al. (2014) have shown that the expression of *cspA* is higher after pretreatment at 10 °C, compared to 4 °C, while expression levels of *hsp60* after pretreatment at 37 °C exceed the values obtained at 45 °C.

Two ornithine decarboxylases were found in *L. delbr.* subsp. *bulgaricus* that are implicated in its acid tolerance (Van De Guchte et al., 2006; El Kafsi et al., 2014). In the present study we found that one of them, *ornB* is differentially expressed in strain LBB.B5 with a maximum of expression at 5 h of incubation when the acidification of the medium has reached 5.0. This pH value might function as a threshold for activating the acid tolerance mechanisms in this species. Such a suggestion is in good agreement with Streit et al. (2008), who have found that in *L. delbr.* subsp. *bulgaricus* CFL1 11 out of 167 proteins, detected on two-dimensional electrophoresis gels, increased in intensity after acidification from pH 6.0 to pH 5.25, including three proteins that corresponded to the stress protein synthesis pathway.

Two genes, related to the threonine synthesis pathway, *thrB* and *thrC* showed the same time pattern in their expression as *ornB* with the highest expression levels at 5h of incubation. However, there is limited evidence that in *L. delbr.* subsp. *bulgaricus de-novo* synthesis of amino-acids is related to acid tolerance (Li et al., 2020). On the other hand, the conservation of the threonine synthesis pathway in this species (Liu et al., 2012; El Kafsi et al., 2014) suggests that this amino-acid may be involved in other processes beside polypeptide production, moreover that in the present study the expression of the *thrB* and *thrC* was found to vary with time and pH. Other studies have shown that levels of intracellular aspartate, glutamate, and alanine increase with the addition of NaCl to the medium as part of the cell's response to osmotic stress (Kets et al., 1996). The fact that in *L. delbr.* subsp.

bulgaricus LBB.B5 maximal expression levels of *thrB* and *thrC* occur during the transition of cells to stationary phase, suggests that threonine synthesis may also be a stress-response and adaptation mechanism in this species.

5. Conclusions

The upregulation of threonine biosynthesis (*thrB* and *thrC*) and a cold-shock protein (*cspA*), with the onset of the stationary phase suggests that threonine and CspA have a function different form just serving the amino-acid anabolism or managing cold stress. Rather, together with the activity of ornithine decarboxylase (*ornB*), threonine and CspA may serve as factors facilitating the transition of the cells to stationary phase and/or adaptation to acidic conditions. This study confirmed that the use of *cspA*, *hsp60*, *ornB*, *thrB* and *thrC* as temporal gene expression markers in *L. delbrueckii* subsp. *bulgaricus* LBB.B5 allows the monitoring of the fermentation process in order to obtain cells in optimal physiological state for the production of starter cultures.

References

Brandi, A., Spurio, R., Gualerzi, C. O., & Pon, C. L. (1999). Massive presence of the *Escherichia coli* 'major cold-shock protein'CspA under non-stress conditions. *The EMBO Journal*, *18*, 1653-1659. doi:10.1093/emboj/18.6.1653

Broadbent, J. R., Oberg, C. J., Wang, H., & Wei, L. (1997). Attributes of the heat shock response in three species of dairy *Lactobacillus*. *Systematic and Applied Microbiology*, *20*(1), 12-19. doi:10.1016/S0723-2020(97)80043-4

Bukau, B., & Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, *92*(3), 351-366.

Dijkstra, A. R., Alkema, W., Starrenburg, M. J., Hugenholtz, J., van Hijum, S. A., & Bron, P. A. (2014). Fermentation-induced variation in heat and oxidative stress phenotypes of *Lactococcus lactis* MG1363 reveals transcriptome signatures for robustness. *Microbial Cell Factories*, *13*, 1-11. doi:10.1186/s12934-014-0148-6

El Kafsi, H., Binesse, J., Loux, V., Buratti, J., Boudebbouze, S., Dervyn, R., ... & van de Guchte, M. (2014). *Lactobacillus delbrueckii* ssp. *lactis* and ssp. *bulgaricus*: a chronicle of evolution in action. *BMC Genomics*, *15*, 1-12. doi:10.1186/1471-2164-15-407

Fonseca, F., Cenard, S., Passot, S. (2015). Freeze-Drying of Lactic Acid Bacteria. In: Wolkers, W., Oldenhof, H. (eds) Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology, vol 1257. Springer, New York, NY. doi:10.1007/978-1-4939-2193-5_24

Goldstein, J., Pollitt, N. S., & Inouye, M. (1990). Major cold shock protein of *Escherichia coli*. *Proceedings of the National Academy of Sciences*, *87*(1), 283-287. doi:10.1073/pnas.87.1.283

Kets, E., Teunissen, P., & De Bont, J. (1996). Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Applied and Environmental Microbiology*, *62*(1), 259-261. doi:10.1128/aem.62.1.259-261.1996

Kim, W. S., Khunajakr, N., Ren, J., & Dunn, N. W. (1998). Conservation of the major cold shock protein in lactic acid bacteria. *Current Microbiology*, *37*, 333-336. doi:10.1007/s002849900387

Kondratenko, M., Kondareva, S., Gyosheva, B., Vlaykovska, A., Shishkova, I., Toteva, N., Goranova, L. (1979). Method for obtaining combination starters for Bulgarian yoghurt. *U.S. Patent* 4156019.

Li, W., Yang, L., Nan, W., Lu, J., Zhang, S., Ujiroghene, O. J., ... & Lv, J. (2020). Whole-genome sequencing and genomic-based acid tolerance mechanisms of *Lactobacillus delbrueckii* subsp. *bulgaricus* LJJ. *Applied Microbiology and Biotechnology*, *104*, 7631-7642. doi:10.1007/s00253-020-10788-5

Lim, E. M., Ehrlich, S. D., & Maguin, E. (2000). Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. *ELECTROPHORESIS: An International Journal*, *21*(12), 2557-2561. doi:10.1002/1522-2683(20000701)21:12<2557::AID-ELPS2557>3.0.CO;2-B

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402-408. doi:10.1006/meth.2001.1262

Liu, E., Hao, P., Konno, T., Yu, Y., Oda, M., Zheng, H., & Ji, Z. S. (2012). Amino acid biosynthesis and proteolysis in *Lactobacillus bulgaricus* revisited: A genomic comparison. *Computational Molecular Bioscience*, *2*, 61-77. doi:10.4236/cmb.2012.23006

Masek, T., Vopalensky, V., Suchomelova, P., & Pospisek, M. (2005). Denaturing RNA electrophoresis in TAE agarose gels. *Analytical Biochemistry*, *336*(1), 46-50. doi:10.1016/j.ab.2004.09.010

Mullan, W.M.A. (2006). Use of starter concentrates in fermented dairy product manufacture. [On-line]. Available from: https://www.dairyscience.info/index.php/cheese-starters/108-starter-concentrates.html . Accessed: 9 September, 2024. Updated July 2017.

Rumian, N., Angelov, M., & Tsvetkov, T. S. (1993). Comparative investigations on activity of *Lactobacillus bulgaricus* during lyophilization. *Cryobiology*, *30*(4), 438-442. doi:10.1006/cryo.1993.1044

Serror, P., Dervyn, R., Ehrlich, S. D., & Maguin, E. (2003). sp-like genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* and their response to cold shock. *FEMS Microbiology Letters*, *226*(2), 323-330. doi:10.1016/S0378-1097(03)00594-9

Shao, Y., Gao, S., Guo, H., & Zhang, H. (2014). Influence of culture conditions and preconditioning on survival *of Lactobacillus delbrueckii* subspecies *bulgaricus* ND02 during lyophilization. *Journal of Dairy Science*, *97*(3), 1270-1280. doi:10.3168/jds.2013-7536

Streit, F., Delettre, J., Corrieu, G., & Béal, C. (2008). Acid adaptation of *Lactobacillus delbrueckii* subsp. *bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance. *Journal of Applied Microbiology*, *105*(4), 1071-1080. doi:10.1111/j.1365-2672.2008.03848.x

Urshev, Z., Hajo, K., Lenoci, L., Bron, P. A., Dijkstra, A., Alkema, W., ... & van Hijum, S. A. (2016). Draft Genome Sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* LBB. B5. *Genome Announcements*, *4*(5), e01090-16. doi:10.1128/genomeA.01090-16.

Van De Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., ... & Maguin, E. (2006). The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences*, *103*(24), 9274-9279. doi:10.1073/pnas.0603024103

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E. (2002). Stress responses in lactic acid bacteria. In: Siezen, R.J., Kok, J., Abee, T., Schasfsma, G. (eds) Lactic Acid Bacteria: Genetics, Metabolism and Applications. Springer, Dordrecht. doi:10.1007/978-94-017-2029-8_12

Wu, R., Song, X., Liu, Q., Ma, D., Xu, F., Wang, Q., ... & Wu, J. (2016). Gene expression of *Lactobacillus plantarum* FS5-5 in response to salt stress. *Annals of Microbiology*, 66, 1181-1188. doi:10.1007/s13213-016-1199-1

Yungareva, T., & Urshev, Z. (2018). The aggregation-promoting factor in *Lactobacillus delbrueckii* ssp. *bulgaricus*: confirmation of the presence and expression of the *apf* gene and in silico analysis of the corresponding protein. *World Journal of Microbiology and Biotechnology*, *34*, 97. doi:10.1007/s11274-018-2480-1

Zhang, Y., Burkhardt, D. H., Rouskin, S., Li, G. W., Weissman, J. S., & Gross, C. A. (2018). A stress response that monitors and regulates mRNA structure is central to cold shock adaptation. *Molecular Cell*, *70*(2), 274-286. doi:10.1016/j.molcel.2018.02.035