HEAT PRE-EXPOSURE IMPROVED CELL RESISTANCE OF PROBIOTIC LACTICASEIBACILLUS RHAMNOSUS GG UPON THERMAL STRESSES

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ABSTRACT

During the last decade, probiotics have been gaining notoriety in complementary medicine and nutrition. Nonetheless, the food industry faces challenges in maintaining the required quantity of viable cells in products. The primary obstacle linked with probiotics in the food industry lies in their vulnerability to processing conditions, as high temperatures. In this study, the heat resistance of *L. rhamnosus* GG was evaluated upon various thermal stress conditions following pre-exposure to high temperature during cultivation, with the objective of innovating the formulation of new functional foods. The strain had two different growth temperature conditions: 37°C (non-exposure) and 45 °C (pre-exposure). To evaluate the heat resistance in both conditions, samples were exposed to high levels of heat challenge at 60, 80, and 100°C for durations of 1, 30, and 60 minutes. The results showed that heat pre-exposed strain had more resistance to thermal stress than heat non-exposed.This occurs because thermal exposure induces changes in bacterial metabolism, enabling them to adapt to the new challenging conditions. Due to its effective thermal resistance in probiotics, pre-exposure proves to be a beneficial strategy for the food industry.

Keywords: Lactobacillus. Thermotolerance. Probiotics.

1 INTRODUCTION

Organizations, such as FAO and WHO, describe probiotics as living microorganisms that when administered in adequate amounts confer health benefits on their hosts[1]. During the last decade, probiotics have been gaining notoriety in complementary medicine and nutrition, supporting in the treatment of intestinal diseases, cancer, hypertension and hypercholesterolemia due to its capacity to modular the intestinal microbiota and enhance the immune system. In the era of functional foods, probiotics are consumable either by adding them to foods or beverages, such as dairy or non-dairy options, or by taking them as supplements[2]. Nonetheless, the food industry faces challenges in maintaining the required quantity of viable cells, which studies indicate to be the effective dose of 10^9 colony-forming units (CFU) per day[3,4,]. The primary obstacle linked with probiotics in the food industry lies in their vulnerability to processing conditions and their sensitivity to gastrointestinal (GI) stresses.

Elevated temperatures represent a key stress factor during the production of various food items like biscuits and bread, where probiotics could be utilized. However, the restricted heat resistance of these microorganisms obstructs their integration into such foods. Exposure to high temperatures causes protein denaturation and subsequent aggregation, among other conditions, impacting the growth, metabolism, and functional attributes of probiotics[5,6]. Acquiring resistance to stress is a crucial physiological requirement for probiotic bacteria to survive heat[7]. Furthermore, *Lacticaseibacillus rhamnosus* GG belongs to the group of lactic acid bacteria (LAB), an established group of microorganisms that have risen as models for studying bacterial stress physiology. Several studies have shown that these bacterias have an adaptive tolerance response that has been instrumental in mitigating adverse effects and enhancing cell viability during subsequent sublethal challenges[8,9,10,]. These microorganisms employ diverse mechanisms, such as conserving cellular energy and altering the composition of the plasma membrane. This is facilitated by synthesizing molecules like chaperones, enzymes, antioxidants, and exopolysaccharides[11,12].

The stress tolerance of LAB can be enhanced through genetic manipulation, pre-exposure to similar stresses (pre-adaptation), or adjustments to cultivation conditions[13]. Moreover, it has been noted that microorganisms resistant to specific stress factors can readily endure the effects of others (cross-adaptation) without compromising their physiological activity[14,15]. In this study, the heat resistance of *L. rhamnosus* GG was evaluated upon various thermal stress conditions following pre-exposure to high temperature during cultivation, with the objective of innovating the formulation of new functional foods.

2 MATERIAL & METHODS

The probiotic strain was *L. rhamnosus* GG (DSM 33156). To perform pre-exposure to the thermal stress, the microorganism was incubated in Man Rogosa and Sharpe broth (MRS) for 24 hours. And it had two different growth temperature conditions: 37°C, the standard temperature growth, and 45°, following the methodology adapted[17]. Afterwards, cells were harvested by centrifugation at 4,500 rpm for 5 min and the cell pellet was homogenized with sterile saline solution 0,9% (w/v). Cell density (OD 595nm) was measured and cell viability was quantified by serial dilution of each sample in sterile 0.9% (w/v) saline solution and spreading them on MRS agar plates. The plates were then aerobically incubated at 37°C for 48 hours, and the colony-forming units per milliliter (CFU/mL) were counted to determine the viability. All the samples were lyophilized.

For evaluation of the heat resistance by pre-exposure to the thermal stress, 0,1g of lyophilized cells (pre-exposed and non-exposed) was resuspended in 0,9 ml of sterile saline solution and then exposed to high levels of heat challenge at 60, 80, and 100°C for durations of 1, 30, and 60 minutes, then cell resistance was determined after heat challenge. The results were expressed as a percentage of viable cells and the data were statistically compared between individuals pre-exposed and unexposed using Student's t-test, with a 95% confidence interval.

3 RESULTS & DISCUSSION

The results indicated that pre-exposed to 45°C during cultivation enhanced the heat resistance of *L. rhamnosus* GG. In the first minute at temperatures of 60°C and 80°C (Table 1), there was no significant difference between the two treatments (pre-exposed and non-exposed). This indicates that for processes found in the food industry, such as rapid pasteurization, the non-exposed strain can withstand rapid temperature-induced stress. However, pre-exposed samples showed statistically significant ($p < 0,05$) improvement at all time points at 100 °C and for 60 minutes at all temperatures, indicating enhanced resistance to thermal stress during subsequent exposures.This approach offers a straightforward and cost-effective solution for the food industry, ensuring that probiotic strains remain less affected during processes involving prolonged exposure to high temperatures, such as slow pasteurization and incorporation into functional products requiring oven baking.

Table 1 Viability (%) of heat pre-exposed (at 45 ºC) and non-exposed (at 37 ºC) *L. rhamnosus GG* under different temperatures (60, 80 e 100 ºC) and times (1, 30 e 60 min) treatments to evaluate thermal resistance.

	Pre-exposed (at 45 ºC)			Non-exposed (at 37 ºC)		
	1 min	30 min	60 min	1 min	30 min	60 min
60 °C	91,1% ±11,87	72,55% ±0,21	46,85% ±6,36 *	70,0% ±0,05	43,1% ±0,08	0% *
80 °C	51,55% ±0,21	57,8% ±2,12 *	47,55% ±9,12 *	38,7% ±0,07	0% *	0% *
100 °C	39,9% ±6,36 *	39,05% ±9,82 *	26% ±0,14 *	0% *	0%	0% *

* means that there is a significant difference ($P > 0,05$) between pre and non-exposed samples by Student's t-test.

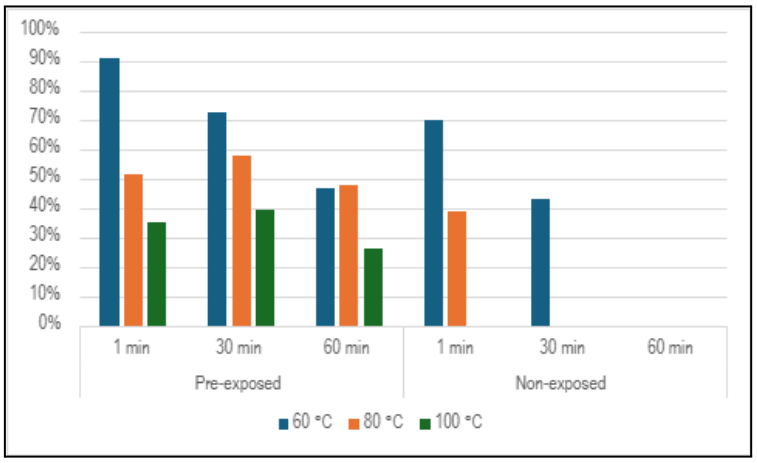

Figure 1 Viability (%) of heat pre-exposed (at 45 ºC) and non-exposed (at 37 ºC) *L. rhamnosus GG* under different temperatures (60, 80 e 100 ºC) and times (1, 30 e 60 min) treatments to evaluate thermal resistance.

As previously mentioned, pre-exposure of the microorganism to sublethal heat makes it more readily adaptable to thermal stress. Proteomic and transcriptome studies of LAB indicate alterations in metabolism upon exposure to thermal and acid stress[15,16]. These modifications result in the stimulation of additional energy production and a decrease in stress levels. A notable example of this is the increased expression of glycolytic enzymes without a concurrent rise in lactic acid production during thermal stress. They also enhance the synthesis of basic compounds (e.g., lysine and diacetyl/acetoin), energy-rich intermediates (ATP and NADH), EPS, and/or glycogen[7,11].

4 CONCLUSION

Pre-exposure to heat demonstrated efficacy in increasing the heat resistance of *L. rhamnosus* GG due to the metabolic changes that occur in these microorganisms during heat stress. Given its stability observed above 100 °C, further research is needed to validate its efficacy at even higher temperatures. This simple and effective method offers food industries an opportunity to develop novel food products for consumers.

REFERENCES

1 Munir, A., Javed, G. A., Javed, S., & Arshad, N. (2022). Levilactobacillus brevis from carnivores can ameliorate hypercholesterolemia: In vitro and in vivo mechanistic evidence. Journal of Applied Microbiology, 133(3), 1725–1742. doi:10.1111/jam.15678.

2 Fusco, A., Savio, V., Cimini, D., D'Ambrosio, S., Chiaromonte, A., Schiraldi, C., & Donnarumma, G. (2023). In vitro evaluation of the most active probiotic strains able to improve the intestinal barrier functions and to prevent inflammatory diseases of the gastrointestinal system. Biomedicines, 11(3), 865. doi:10.3390/biomedicines11030865

3 Latif, A., Shehzad, A., Niazi, S., Zahid, A., Ashraf, W., Iqbal, M. W., … Korma, S. A. (2023). Probiotics: mechanism of action, health benefits and their application in food industries. Frontiers in Microbiology, 14, 1216674. doi:10.3389/fmicb.2023.1216674

4 Stavropoulou, E., & Bezirtzoglou, E. (2020). Probiotics in medicine: A long debate. Frontiers in Immunology, 11, 2192. doi:10.3389/fimmu.2020.02192

5 Van De Guchte, M. (2002). Stress responses in lactic acid bacteria. Em: Lactic Acid Bacteria: Genetics, Metabolism and Applications. Em: Lactic Acid Bacteria: Genetics, Metabolism and Applications, 187–216.

6 Ma, J., Xu, C., Liu, F., Hou, J., Shao, H., & Yu, W. (2021). Stress adaptation and cross-protection of Lactobacillus plantarum KLDS 1.0628. CyTA - Journal of Food, 19(1), 72–80. doi:10.1080/19476337.2020.1859619

7 Zhang, C., Gui, Y., Chen, X., Chen, D., Guan, C., Yin, B., … Gu, R. (2020). Transcriptional homogenization of Lactobacillus rhamnosus hsryfm 1301 under heat stress and oxidative stress. Applied Microbiology and Biotechnology, 104(6), 2611–2621. doi:10.1007/s00253-020-10407-3

8 Aakko, J., Sánchez, B., Gueimonde, M., & Salminen, S. (2014). Assessment of stress tolerance acquisition in the heat-tolerant derivative strains of Bifidobacterium animalis subsp. lactis BB-12 and Lactobacillus rhamnosus GG. Journal of Applied Microbiology, 117(1), 239–248. doi:10.1111/jam.12520.

9 Yang, Y., Huang, S., Wang, J., Jan, G., Jeantet, R., & Chen, X. D. (2017). Mg2+ improves the thermotolerance of probiotic Lactobacillus rhamnosus GG, Lactobacillus casei and Lactobacillus plantarum P-8. Letters in Applied Microbiology, 64(4), 283–288. doi:10.1111/lam.12716.

10 Moon, S., Ham, S., Jeong, J., Ku, H., Kim, H., & Lee, C. (2023). Temperature matters: Bacterial response to temperature change. The Journal of Microbiology, 61(3), 343–357. doi:10.1007/s12275-023-00031-x

11 Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., … Kok, J. (2016). Stress physiology of Lactic acid bacteria. Microbiology and Molecular Biology Reviews: MMBR, 80(3), 837–890. doi:10.1128/mmbr.00076-15

12 Derunets, A. S., Selimzyanova, A. I., Rykov, S. V., Kuznetsov, A. E., & Berezina, O. V. (2024). Strategies to enhance stress tolerance in lactic acid bacteria across diverse stress conditions. World Journal of Microbiology and Biotechnology, 40(4), 126. doi:10.1007/s11274-024-03905-3

13 Belenky P, Collins JJ (2011) Microbiology. Antioxidant strategies to tolerate antibiotics. Science 334(6058):915–916. https:// doi. org/ 10.1126/science.1214823.

14 Hao, F., Fu, N., Ndiaye, H., Woo, M. W., Jeantet, R., & Chen, X. D. (2021). Thermotolerance, survival, and stability of lactic acid bacteria after spray drying as affected by the increase of growth temperature. Food and Bioprocess Technology, 14(1), 120–132. doi:10.1007/s11947-020-02571-1

15 Koponen J, Laakso K, Koskenniemi K, Kankainen M, Savijoki K, Nyman TA, de Vos WM, Tynkkynen S, Kalkkinen N, Varmanen P. 2012. Effect of acid stress on protein expression and phosphorylation in Lactobacillus rhamnosus GG. J Proteomics 75:1357–1374. http://dx.doi .org/10.1016/j.jprot.2011.11.009.

16 Heunis T, Deane S, Smit S, Dicks LM. 2014. Proteomic profiling of the acid stress response in Lactobacillus plantarum 423. J Proteome Res 13: 4028–4039. http://dx.doi.org/10.1021/pr500353x.

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