SCREENING AND DEVELOPMENT OF THERMO– AND ACID–TOLERANT PROBIOTIC LACTIC ACID BACTERIA USING ADAPTIVE LABORATORY EVOLUTION

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DOI: 10.54503/978-9939-481-23-4-469

Abstract

The development of stress–tolerant probiotic lactic acid bacteria (LAB) is essential for ensuring their viability during industrial processing and gastrointestinal passage. In this study, a comprehensive selection strategy was applied to identify

LAB strains with enhanced resistance to technological and physiological stress factors, during the collection of 96 LAB strains, a systematic screening was established, resulting in the selection of 29 probiotic strains exhibiting increased stress tolerance. Targeted selection using of Adaptive laboratory evolution method was applied to obtain heat– and acid–resistant strains, followed by evaluation of their viability under spray drying conditions and low pH. Furthermore, the combined effects of thermal and acidic stresses were investigated, resulting in the identification of 8 dual–stress–tolerant (acid– and heat–resistant) LAB mutants. These strains demonstrate strong potential for microencapsulation and represent promising candidates for the development of stable and effective probiotic formulations.

Keywords and phrases: Lactic acid bacteria; probiotics; Adaptive laboratory evolution; heat tolerance; acid tolerance.

ՋԵՐՄԱ- ԵՎ ԹԹՎԱԿԱՅՈՒՆ ՊՐՈԲԻՈՏԻԿ ԿԱԹՆԱԹԹՎԱՅԻՆ ԲԱԿՏԵՐԻԱՆԵՐԻ ՍՔՐԻՆԻՆԳ ԵՎ ՍՏԱՅՈՒՄ՝ ԱԴԱՊՏԻՎ ԼԱԲՈՐԱՏՈՐ ԷՎՈԼՅՈՒՑԻԱՅԻ ՄԵԹՈԴԻ ԿԻՐԱՌՄԱՄԲ

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Համառոտագիր

Սթրեսակայուն պրոբիոտիկ կաթնաթթվային բակտերիաների (ԿԹԲ) զարգացումը կարևոր նշանակություն ունի արդյունաբերական մշակման և մարսողական համակարգով անցման ընթացքում դրանց կենսունակության ապահովման համար: Սույն ուսումնասիրությունում կիրառվել է համալիր սելեկցիոն ռազմավարություն՝ տեխնոլոգիական և ֆիզիոլոգիական սթրեսային գործոնների հանդեպ բարձր դիմադրողականություն ունեցող ԿԹԲ շտամների հայտնաբերման նպատակով: Ուսումնասիրության ընթացքում ձևավորվել և համակարգված սքրինինգի է ենթարկվել 96 ԿԹԲ շտամներից բաղկացած հավաքածու, որի արդյունքում ընտրվել են 29 պրոբիոտիկ շտամներ՝ բարձր սթրեսակայունությամբ: Թիրախային սելեկցիայի և միկրոսելեկցիայի

մեթոդների կիրառմամբ ստացվել են ջերմակայուն և թթվակայուն շտամներ, որոնց կենսունակությունը գնահատվել է փոշեցիր չորացման պայմաններում: Բացի այդ՝ ուսումնասիրվել է ջերմային և թթվային սթրեսների համակցված ազդեցությունը, որի արդյունքում հայտնաբերվել են երկակի կայունությամբ (թթվակայուն և ջերմակայուն) օժտված 8 ԿԹԲ մուտանտներ: Նշված շտամները ցուցաբերում են բարձր պոտենցիալ միկրոպատիճավորման համար և խոստումնալից թեկնածուներ են՝ կայուն և արդյունավետ պրոբիոտիկ պատրաստուկների մշակման համար:

Բանալի բառեր և բառակապակցություններ՝ կաթնաթթվային մանրէներ, պրոբիոտիկներ, ադապտիվ լաբորատոր էվոլյուցիա, ջերմակայունություն, թթվակայունություն:

СКРИНИНГ И ПОЛУЧЕНИЕ ТЕРМО- И КИСЛОТОУСТОЙЧИВЫХ ПРОБИОТИЧЕСКИХ МОЛОЧНОКИСЛЫХ БАКТЕРИЙ С ИСПОЛЬЗОВАНИЕМ МЕТОДА АДАПТИВНОЙ ЛАБОРАТОРНОЙ ЭВОЛЮЦИИ

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Аннотация

Разработка стрессоустойчивых пробиотических молочнокислых бактерий (МКБ) имеет важное значение для обеспечения их жизнеспособности в процессе промышленной переработки и прохождения через желудочно-кишечный тракт. В данном исследовании была применена комплексная стратегия селекции для выявления штаммов МКБ с повышенной устойчивостью к технологическим и физиологическим стрессовым факторам. В ходе сбора было создано и систематически протестировано 96 штаммов МКБ, в результате чего было отобрано 29 пробиотических штаммов, демонстрирующих повышенную стрессоустойчивость. Для получения термо- и кислотоустойчивых штаммов

были применены методы целенаправленной селекции и микроселекции, после чего была проведена оценка их жизнеспособности в условиях распылительной сушки. Кроме того, были исследованы комбинированные эффекты термического и кислотного стресса, что привело к идентификации 8 мутантов МКБ, обладающих двойной стрессоустойчивостью (кислото– и термоустойчивостью). Эти штаммы демонстрируют большой потенциал для микрокапсулирования и представляют собой перспективные кандидаты для разработки стабильных и эффективных пробиотических препаратов.

Ключевые слова и фразы: молочнокислые бактерии; пробиотики; адаптивная лабораторная эволюция; термостойкость; кислотоустойчивость.

Intaduction

Lactic acid bacteria (LAB) are widely used in the food, pharmaceutical, and nutraceutical industries due to their technological functionality and health–promoting properties. They play a key role in the production of fermented foods, contribute to sensory characteristics, enhance nutritional value, and, in the case of probiotic strains, exert beneficial effects on host health **[4, 6, 8].** For LAB to be effective in industrial applications and as probiotics, they must maintain viability and functional activity throughout processing, storage, and passage through the gastrointestinal tract.

During industrial manufacturing and subsequent use, LAB are exposed to a variety of environmental and physiological stress factors, including high and low temperatures, osmotic pressure, acidic conditions, bile salts, oxidative stress, and dehydration processes such as freeze–drying or spray–drying. These stress conditions can significantly reduce cell viability, metabolic activity, and stability, ultimately limiting the efficiency and reliability of LAB–based products **[2, 3, 7, 9].** Therefore, stress tolerance is considered a critical selection criterion for technologically and functionally valuable LAB strains.

The ability of LAB to survive and adapt to stress is strain–dependent and associated with specific cellular and molecular mechanisms, such as membrane composition changes, synthesis of stress proteins, activation of protective metabolic pathways, and regulation of intracellular pH **[7].** Evaluating stress tolerance allows for the identification of robust strains capable of withstanding adverse conditions without loss of desirable technological or probiotic properties **[1].**

In this context, the evaluation of stress tolerance in selected lactic acid bacteria is essential for the development of high–performance starter cultures and probiotic preparations. Systematic assessment of LAB resistance to technological and physiological stressors provides valuable insights into their adaptive potential. It supports the rational selection of strains suitable for industrial processing and functional food applications **[1, 5, 8].**

Material and Methods

Formation of Lactic Acid Bacteria Collection: In the first stage of the study, lactic acid bacteria (LAB) strains were isolated from various sources and subjected to biological activation. Preliminary evaluation was performed based on cell viability and growth characteristics. As a result, an initial collection consisting of 96 LAB strains was established and used for subsequent screening and selection procedures.

Evaluation of Thermal Tolerance: Thermal tolerance of LAB strains was assessed by examining their growth ability at 45, 50, and 55 °C. To evaluate tolerance to extreme temperatures, cultures were exposed to heat shock at 55–80 °C for 1 hour, followed by incubation under optimal conditions (37 °C for 18–24 h). Growth was then assessed to determine strain survival and tolerance.

Evaluation of Acid Tolerance: Acid tolerance was determined by assessing LAB growth and viability at different pH levels (pH 5.5, 4.5, 3.5, 2.5, and 1.5). Bacterial cultures were exposed to acidic conditions for 1 hour and subsequently incubated at 37 °C. Growth intensity was used as an indicator of acid resistance.

Screening and use of the Adaptive Laboratory Evolution (ALE) method of Stress-Tolerant Strains: Before subjecting LABs to ALE, bacteria were screened for thermotolerance across different temperatures. After this screening, ALE was performed in a manner similar to that described by Jeon et al. [1]: heat exposure at 75 –C, followed by cultivation at 37 –C for 24 h, repeated for 60 days. Targeted screening was performed on the most thermotolerant and acid-tolerant strains using sequential subculturing under non-permissive conditions. For thermotolerant microselection, cultures were heat-shocked at 80 °C for 5 min and then plated on LAPTg agar under optimal growth conditions. For acid-tolerant ALE, cultures were exposed to extremely low-pH environments using the same sequential selection strategy. This process was repeated for multiple passages to enhance stress tolerance.

Determination of LAB viability after spray drying: Bacterial viability before and after spray drying was evaluated by determining colony-forming units (CFU/mL). Dried powder samples were rehydrated in sterile buffer, serially diluted tenfold, and plated on LAPTg agar medium. Plates were incubated at 37 °C for 48 h, after which colonies were counted. Cell survival was calculated using the following equation:

$$\text{Survival (\%)} = (\text{CFU after drying} / \text{CFU before drying}) \times 100$$

Selection of Dual Stress-Tolerant Strains: To identify strains with dual resistance, selected LAB cultures were simultaneously exposed to acid and heat stress. Strains that retained high viability and growth capacity under both stress factors were selected. Based on comparative analysis, a collection of eight LAB strains exhibiting combined acid and thermal tolerance was established for further studies and potential application in microencapsulation and probiotic formulation development.

Statistical Analysis: All data are expressed as mean ± standard error of the mean (SEM) for the respective parameters. Statistical comparisons among groups within each experiment were performed using one-way analysis of variance (ANOVA). Statistical significance was established at $p \leq 0.05$.

Results

Establishment of the LAB Collection

A total of 96 lactic acid bacteria (LAB) strains were successfully collected, activated, and characterised based on growth performance and viability. This collection served as the initial pool for subsequent screening of stress tolerance. Preliminary evaluation revealed considerable strain-dependent variability in growth intensity and survival under non-optimal conditions, highlighting the necessity of targeted selection.

Initial study of the resistance of selected LABs to stress factors

The stability of selected LAB strains to various stress factors inherent in the processes of obtaining, drying, microencapsulation and application of probiotic preparations was studied. The effects of thermal, acid factors were evaluated. As a result, a collection of 29 probiotic strains was selected and formed, demonstrating high stability under various stress factors.

Table 1.

Screening of heat–resistant lactic acid bacteria

Number of strains	LAB growth at 0 °C			
	45	50	55	60
9	+++	+++	+	–
14	+++	+++	–	–
35	+++	++	–	–
5	+++	+++	++	+
33	+++	++	+	–

(+ weak growth, ++ medium growth, +++ good growth, – no growth)

Thermal tolerance screening at 45, 50, and 55 °C demonstrated that the majority of strains grew at 45 °C, with a progressive reduction in growth at higher temperatures. Only a limited number of strains could grow at temperatures of 60 °C, indicating a high level of thermally sensitive isolates. Fourteen strains exhibited high growth capacity (+++ to ++) at both 50 and 55 °C and were therefore classified as thermoresistant.

Table 2.

Screening of acid–resistant lactic acid bacteria

Number of strains	Growth at pH				
	5.5	4.5	3.5	2.5	1.5
5	+++	+++	+	–	–
37	+++	+++	–	–	–
44	+++	++	–	–	–
6	+++	+++	+	–	–
4	+++	++	+	–	–

(+ weak growth, ++ medium growth, +++ good growth, – no growth)

AciResistance assays revealed that most LAB strains grew well at pH 5.5 and 4.5; however, growth declined markedly at pH ≤3.5. Fifteen strains retained measurable growth at pH 3.5.

Determination of the thermotolerant profile of LABs

Study of the tolerance of isolated strains to extreme (non–permissive) temperatures. After 1 hour of exposure to extreme temperatures, the LAB cultures

were incubated at 37– for 18 hours, and the presence of growth was checked.

Table 3.

Viability of LAB strains after treatment at shock temperatures

Number of strains	Survival at extreme temperatures, 0 °C				
	55	60	65	70	80
2	+++	+++	+	+	–
3	+++	+++	+	–	–
4	+++	++	–	–	–
2	+++	+++	+++	+	+
3	+++	++	+	–	–

(+ weak growth, ++ medium growth, +++ good growth, – no growth)

Applying ALE under non–permissive conditions significantly enhanced stress tolerance. Heat–adapted strains derived from four highly thermotolerant parental strains demonstrated improved survival following short–term heat shock at temperatures of 70–80 °C. In total, thermotolerant mutants were obtained, among which selected representatives displayed stable growth following extreme thermal treatment.

Similarly, acid–adapted mutants obtained from four acid–tolerant strains exhibited increased survival at low pH levels compared to their parental strains. The repeated selection cycles resulted in mutants capable of maintaining viability under highly acidic conditions that were lethal to the original isolates.

The acid tolerance profiles of LABs were studied. After exposure to an acidic environment, the pH of the cultures was adjusted to neutral pH and incubated at 37°C for 18 hours.

Table 4.

Stability of LAB heat–resistant mutants (LABs) after 5 minutes of exposure to extreme temperatures

TT number	Survival at extreme temperatures, °C				
	60	70	75	80	85
4	+++	+++	++	+	–
8	+++	++	+	±	–
13	+++	++	±	–	–

(+ weak growth, ++ medium growth, +++ good growth, – no growth)

To maintain species diversity, ALE was carried out in all 4 heat–resistant strains (TT1 – TT4)

19 heat–resistant mutants were isolated from the TT1 mutant, 16 from the TT2 mutant, 14 from the TT3 mutant, and 12 from the TT4 mutant. 2 mutants from each group were isolated for further studies.

Screening of aci-tolerant LABs

The acid tolerance profiles of LABs were studied. LAB cultures were incubated for 48 hours after being kept at low pH for 1 hour and checked for growth. After exposure to an acidic environment, the pH of the cultures was adjusted to neutral pH and incubated at 37°C for 18 hours.

Table 5.

Tolerance of LAB strains to extreme pH

Number of strains	Tolerance to pH			
	3.5	3.0	2.5	1.5
4	+++	++	+	–
5	+++	++	±	–
6	+++	+	–	–

(+ low tolerant, ++ medium tolerant, +++ high tolerant, ± not all strains are tplerant, – not tolerant)

Four acid-tolerant (AT) lactic acid bacteria strains were identified. ALE methodology was used to enhance acid- and thermotolerance of the identified LAB. strains. As a result of using the ALE method, it was possible to achieve heat and acid resistance of lactic acid bacteria at a level of 85°C and pH 2.

Cross-selection of lactic acid bacteria strains with dual acid- and heat-tolerance

A test of simultaneous exposure to dual stress conditions was performed, during which the strains were cultivated under combined conditions of low pH and high temperature of achieve more stringent selection.

Combined exposure to acid and heat stress resulted in a more stringent selection pressure. Only eight LAB strains maintained high viability and growth capacity under the simultaneous influence of low pH and elevated temperature. These dual stress-tolerant strains (acid- and thermotolerant) represent the most robust candidates identified in this study and are considered promising for further application in microencapsulation processes and the development of stable probiotic preparations.

Viability of Stress Tolerant (TAT) LABs During Spray Drying

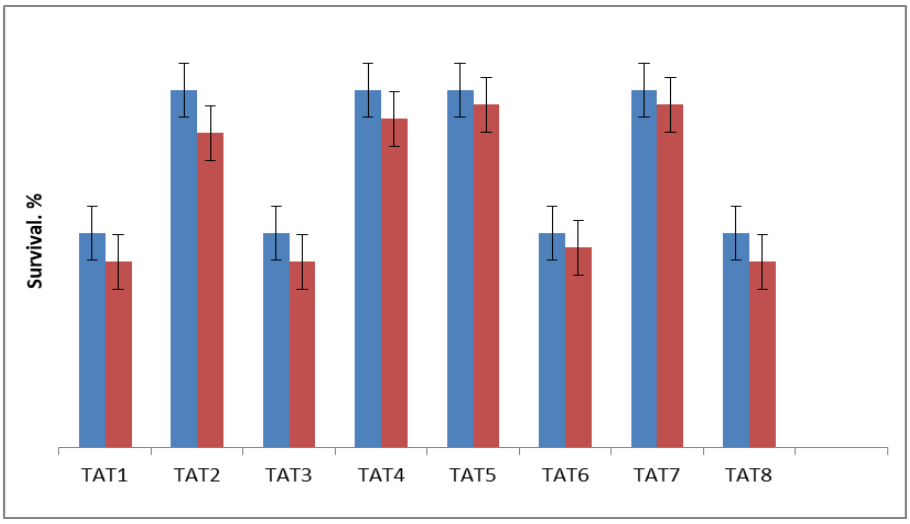

Figure 1. Strain viability after spray drying.
The blue columns – Ale–adapted strains. The red columns–parental strains.

The data presented in Figure 1 demonstrate that spray drying resulted in a reduction of viable cell counts in all tested strains, which is expected due to the combined effects of heat and dehydration stress. However, the extent of viability loss was clearly strain–dependent. Stress–tolerant (TAT) and ALE–adapted strains retained a significantly higher proportion of viable cells compared to their parental counterparts, indicating that prior adaptation to thermal and acidic stress enhances resistance to spray–drying conditions. These findings confirm that thermal and acid tolerance are important selection criteria for developing robust probiotic strains suitable for industrial processing. The selected dual stress–tolerant strains represent promising candidates for spray–dried and microencapsulated probiotic formulations with improved stability and survival.

This work was supported by the Science Committee of the Ministry of Education, Science, Culture and Sports of the Republic of Armenia within the framework of the Scientific Productivity Promotion Grant Program (Grant No. 25RG–1F070).

References

1. BommasamudramJ.,Kumar P.,Kapur S.,SharmaD., Devappa S.,Development of Thermotolerant Lactobacilli Cultures with Improved Probiotic Properties Using Adaptive Laboratory Evolution Method, Probiotics Antimicrob Proteins. 2023. Aug;15(4):832–843. DOI: 10.1007/s12602–021–09892–3
2. Carvalho A. S., Silva J., Ho P., Teixeira P., Malcata F. X., & Gibbs P. Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze–dried Lactobacillus delbrueckii ssp. bulgaricus. Biotechnology Progress, 2004. 20(1), 248–254.
3. Corcoran B. M., Ross R. P., Fitzgerald G. F., & Stanton C. Comparative survival of probiotic lactobacilli spray–dried in the presence of prebiotic substances. Journal of Applied Microbiology, 2004. 96(5), 1024–1039
4. FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organisation of the United Nations / World Health Organisation, 2002. London, Ontario, Canada.
5. Hovhannisyan H.G., Pashayan M.M., Barseghyan A.H., Grigoryan G.G., Gaboyan E. H., Danielyan L. V., Health Promoting Potentials of Armenian Functional Sour Milk "NARINE" and its Starter

Lactobacillus helveticus MDC 9602. The New Armenian Medical Journal, V.17, 2023, Issue 3, p. 66–73, DOI: https://doi.org/10.56936/18290825–2023.17.3–66

6. International Scientific Association for Probiotics and Prebiotics. The ISAPP consensus statement on the definition and scope of postbiotics. Nature Reviews Gastroenterology & Hepatology, 2021, 18, 649–667.
7. Papadimitriou, K., Alegría Á., Bron P. A., et al. Stress physiology of lactic acid bacteria. Microbiology and Molecular Biology Reviews, 2016. 80(3), 837–890.
8. Sanders M. E., Merenstein D. J., Reid G., Gibson G. R., & Rastall R. A Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. Nature Reviews Gastroenterology & Hepatology, 2019. 16, 605–616.
9. Saarela M., Virkajärvi I., Alakomi H. L., Sigvart–Mattila P., Mättö J. Stability and functionality of freeze–dried probiotic Bifidobacterium cells during storage in juice and milk. International Dairy Journal, 2006. 16(12), 1477–1482.