Food & Health 2023, 6(1): 30-35

Food & Health Journal

Journal homepage: fh.srbiau.ac.ir

Optimization and modeling of growth conditions of *Lactobacillus brevis* IBRC10818 for biosynthesis of gamma aminobutyric acid, affected by ultrasonic shock

Mahboobe Rezaei [1], Younes Ghasemi [2], Anousheh Sharifan [1*], Hossein Bakhoda [3]

[1] Department of the Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3] Department of Agricultural Mechanization, Faculty of Agriculture and Food Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

ARTICLE INFO

Original Article

Article history:
Received 26 November 2022
Revised 14 February 2023
Accepted 04 April 2023
Available online 20 April 2023

Keywords:
Ultrasonic shock
Lactobacillus brevis
Gamma-aminobutyric acid
Response surface methodology

ABSTRACT

Gamma-aminobutyric acid (GABA) is a crucial inhibitory neurotransmitter in the nervous system. Microbial biosynthesis of GABA has gained attention as an effective production method. This study aimed to optimize and model the production of GABA from *Lactobacillus brevis* IBRC 10818 using an ultrasonic shock and response surface methodology. The factors investigated included ultrasonic intensity, ultrasonic time, incubation period, and monosodium glutamate concentration. The shock was applied during the lag phase of growth. The highest predicted GABA production for *L. brevis* IBRC10818 was 212.2 mg/L, with optimal conditions of 2.98% monosodium glutamate, 70.8 hours of incubation, 6.30 minutes of shock time, and a frequency of 33 kHz. Three replicates were conducted under these optimal conditions, resulting in an actual GABA production of 203.5 mg/L, which closely matched the predicted value. This study demonstrates the successful optimization of growth conditions and validates the accuracy of the model.

1. Introduction

Gamma-aminobutyric acid (GABA) is a non-protein, four-carbon amino acid that was first synthesized in 1883 through the decarboxylation of glutamic acid catalyzed by the glutamic acid decarboxylase enzyme (GAD) (1). GABA is widely recognized as an important compound in the pharmaceutical industry and is considered a bioactive substance in certain beneficial foods, leading to an increased demand for its production (2). This demand is primarily driven by alpha carboxylation in the presence of L-glutamate and the enzyme GAD (3). GABA synthesis occurs in lactic acid bacteria through fermentation (4). GABA synthesis occurs in lactic acid bacteria through fermentation, with various species such as *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* known as good producers of GABA (5). These bacteria, often referred to as "probiotics," produce Generally Recognized as Safe (GRAS) products for therapeutic use in humans (6). The production of GABA by lactic acid bacteria is influenced by several environmental conditions, including glutamate concentration, temperature, and pH (7). GABA exhibits bioactive potential and plays a crucial role in the function of the sympathetic nervous system. It has been used in the treatment of Alzheimer's disease, schizophrenia, and multiple sclerosis and has shown effectiveness in regulating blood pressure, heart rate, emotions, sleep quality, pain relief, and inflammation reduction (8, 9). Additionally, GABA stimulates insulin secretion from the pancreas and possesses anti-diabetic and anti-cancer properties (9). Due to its medical importance, GABA has gained attention in medicine and the food industry (10). Although GABA is naturally present in sources such as potatoes, spinach, broccoli, tomatoes, apples, grapes, barley, and corn, the amounts are insufficient to fulfil its therapeutic potential (11). Physical treatments, such as ultrasonic shock, have been utilized to promote the survival of living cells by exerting sub-lethal effects on the cell membrane. Ultrasound-induced cavitation increases microbial cell permeability,

*Corresponding author: Department of the Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

E-mail address: sharifan.anousheh2022@gmail.com (Anousheh Sharifan).

enhancing material transport and potentially influencing growth, proliferation, and metabolic processes (12). Microorganisms adapt to changes in their environment to optimize growth conditions, which can cause stress, growth retardation, or a prolonged lag phase. However, microorganisms can also adapt to these conditions, improving their efficiency and production of survival-related materials, thus enhancing resistance to unstable and stressful environmental conditions (5). While previous studies have focused on optimizing GABA production, this research evaluates explicitly the growth conditions of *Lactobacillus brevis* IBRC10818 for the biosynthesis of GABA under the influence of ultrasonic shock.

2. Materials and methods

2.1. Materials

Lactobacillus brevis IBRC (10818) (Iran's Center for Genetic Resources, Iran) named *L. brevis* for briefness, MRS Broth culture medium (Liofilchem, Italy), MRS Agar culture medium (Merck, Germany), Gamma-aminobutyric acid (Sigma Aldrich, Germany). All other chemicals used were of analytical grade and were provided by Merck Chemical Co. (Darmstadt, Germany).

2.2. Preparation of pre-culture

A number of colonies were transferred from an active culture of *L. brevis* to the MRS broth culture medium and incubated at 37 °C for 24 hours to obtain a cell mass. The cell mass was then stored at -80 °C in stocks containing 20% glycerol. To prepare the inoculum for the experiments, several colonies were also added to a 10 ml sterile standard MRS broth container (13).

2.3. Microbial inoculation

For the initial inoculation, bacterial cultures were centrifuged at 8000 rpm for 10 minutes. The microbial suspension obtained was used as an inoculum at a concentration of 4% (v/v) in 15 ml sterile tubes (14).

2.4. Ultrasonic shock

During the lag phase, the bacteria were subjected to ultrasonic shock using an ultrasonic device with a probe tip measuring 1.27 × 1.27 cm. The temperature was maintained at 10 °C using crashing ice during ultrasonication, and then the samples were moved to a 37 °C incubator for further incubation (15).

2.5. Determination of GABA generated by UV/vi's spectrophotometer

To determine the amount of GABA produced, 0.2 ml of 0.2 M borate buffer (pH 8.0) and 1 mL of 6% phenol reagent solution were mixed with 0.1 ml of microbial supernatant. The microbial supernatant was obtained by centrifuging the microbial culture for 15 minutes at 4000 rpm at 22 °C. The standard GABA's respective volumes were 3 ml and 1 mL. Then, 0.4 ml of 7.5% sodium hypochlorite was added, and the test tubes were boiled for 10 minutes. After boiling, the tubes were rapidly cooled in water and ice for 5 minutes. The resulting absorbance was measured at 630 nm using a spectrophotometer. A calibration curve was prepared using a standard solution of 500 ppm GABA to determine the amount of GABA (16).

2.6. Response surface methodology

The study utilized Design Expert software (version 12.03) for optimization. The following independent variables were considered: ultrasonic intensity (25, 32.5, and 40 kHz), ultrasonic time (5, 12, and 20 minutes), incubation time (15, 43.5, and 72 hours), and the amount of monosodium glutamate (1, 5.5, and 10 g/L). In addition to several independent variables, one dependent variable (GABA) was used in the study (Table 1). Nineteen tests were conducted for the ultrasonic shock using the independent variables at three distinct levels (-1, 0, +1), and the relative impact of two independent variables on the response was examined using 3D plots.

Table 1. Factors and variables tested based on ultrasonic shock.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	MSG	g/L	Numeric	1.0000	10.00	-1 ↔ 1.00	+1 ↔ 10.00	5.50	3.35
B	Incubation time	hrs.	Numeric	15.00	72.00	-1 ↔ 15.00	+1 ↔ 72.00	43.50	21.24
C	Time of ultrasonic shock	min	Numeric	5.00	20.00	-1 ↔ 5.00	+1 ↔ 20.00	12.50	5.59
D	Frequency		Numeric	25.00	40.00	-1 ↔ 25.00	+1 ↔ 40.00	32.50	5.59

2.7. Statistical plan

Design Expert software version 12.03 was used for statistical analysis and optimization. The ANOVA method was employed to analyze the variance of the independent variables in the experimental design at a 95% confidence level. The conditions evaluated by the software and the ideal samples were repeated three times, and their averages were compared to the predicted values generated by Design-Expert.

3. Results

The results of the effect of ultrasonic shock on GABA production by *L. brevis* are presented in Table 2, Table 3, and Figure 1. The R^2 values, which indicate the model's goodness of fit, were found to be close to 100%, indicating a strong relationship between the experimental data and the predicted values. This suggests that the proposed model is reliable for predicting GABA production. The following equation was

used to determine the final model for predicting GABA production.

x=137.68+60B-8.9C-75D-88.77AB-18.37AC+26.12AD+16.12BC-28.87BD-13.37CD

The analysis of the variables showed that the amount of monosodium glutamate (MSG) had no significant effect on GABA production within the tested range of 1-10%. Incubation time significantly positively affected GABA production, with the highest production observed at 72 hours of incubation, reaching slightly over 200 mg/L. Shock time also had a significant effect, with longer shock times (20 minutes) leading to a decrease in GABA production to around 140 mg/FL. The frequency of the ultrasonic shock showed a decreasing trend, with higher frequencies (40 kHz) resulting in a decrease in GABA production to approximately 70 mg/L. The interaction of the studied variables was also analyzed. Fig. 2a shows the simultaneous effect of incubation time and MSG on GABA production. The highest GABA production was observed with a long incubation time (72 hours) and a low concentration of MSG (1-2%). Fig. 2b illustrates the simultaneous effect of shock time and MSG on GABA production, indicating a slight increase in GABA production with increasing MSG concentration and a slight decrease with longer shock times.

Table 2. Analysis of variance of GABA production from *L. brevis* affected by ultrasonic shock.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	42357.85	10	4235.78	2765.04	< 0.0001	significant
A:MSG	0.5000	1	0.5000	0.3264	0.5835	
B: Time of incubation	7200.00	1	7200.00	4700.02	< 0.0001	
C: Time of ultrasonic shock	792.10	1	792.10	517.07	< 0.0001	
D: Frequency	11100.50	1	11100.50	7246.19	< 0.0001	
AB	12355.22	1	12355.22	8065.25	< 0.0001	
AC	2701.12	1	2701.12	1763.24	< 0.0001	
AD	1092.02	1	1092.02	712.85	< 0.0001	
BC	2080.13	1	2080.13	1357.87	< 0.0001	
BD	1334.03	1	1334.03	870.83	< 0.0001	
CD	1431.13	1	1431.13	934.21	< 0.0001	
Residual	12.26	8	1.53			
Lack of Fit	9.59	6	1.60	1.20	0.5210	not significant
Pure Error	2.67	2	1.33			
Cor Total	42370.11	18				

Table 3. Fit coefficients for *L. brevis* under the influence of ultrasonic shock.

Std. Dev.	1.24	**R^2**	0.9997
Mean	137.68	**Adjusted R^2**	0.9993
C.V. %	0.8989	**Predicted R^2**	0.9948
		Adeq Precision	166.5515

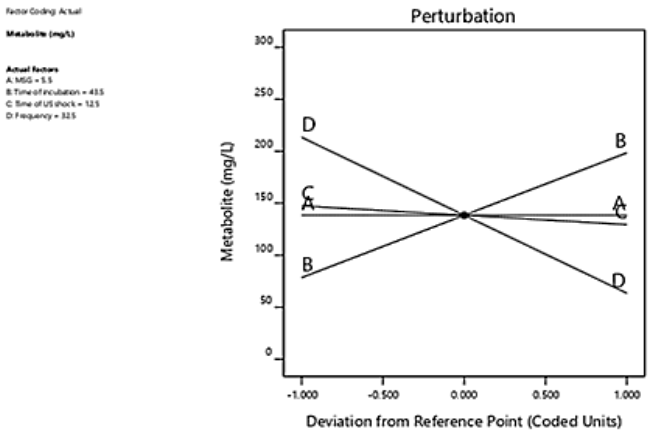

Fig. 1. Simple effects of monosodium glutamate (A), incubation time (B), shock time (C) and frequency (D) on GABA production.

Fig. 2c demonstrates the simultaneous effect of frequency and MSG on GABA production. The highest GABA production was achieved at the lowest frequency (25 kHz) and the highest MSG concentration (10%). Fig. 2d shows the effect of incubation time and shock time on GABA production, indicating that higher incubation times led to increased GABA production, while shock time has a negligible impact. Fig. 2e represents the simultaneous effect of frequency and shock time, showing a decrease in GABA production with increasing frequency but an increase with longer shock times. Overall, these results suggest that the optimal conditions for GABA production by *L. brevis* involve a long incubation time (around 72 hours), a low concentration of monosodium glutamate (1-2%), a short shock time (5 minutes), and a low frequency (25 kHz).

4. Discussion

Fermentation time is one of the critical factors during the fermentation process to produce GABA (17). The temperature dependence for GABA production depends on the type of microorganism and the compounds in the culture medium. According to the results, it can be seen that an incubation time of 72 hours, as an independent variable, is the most suitable time for GABA production. The decrease in GABA production is associated with the death phase of cells and a reduction in cell count (18). Fermentation time is a crucial factor for GABA production, along with temperature and pH. Lb. plantarum DSM19463 and *Lb. paracasei* NFRI 7415 achieved their maximum GABA levels after 72 h and 144 h of fermentation, respectively, with concentrations of 4.83 mM and 60 mM (19, 20). The production of GABA during 72 hours of incubation in this study, at a rate of 203.5 mg/L, was similar to the findings of Komatsuki et al., who reported that *L. plantarum* 19463dsm produced the highest amount of GABA during 72 hours of fermentation (21). GABA production begins in the

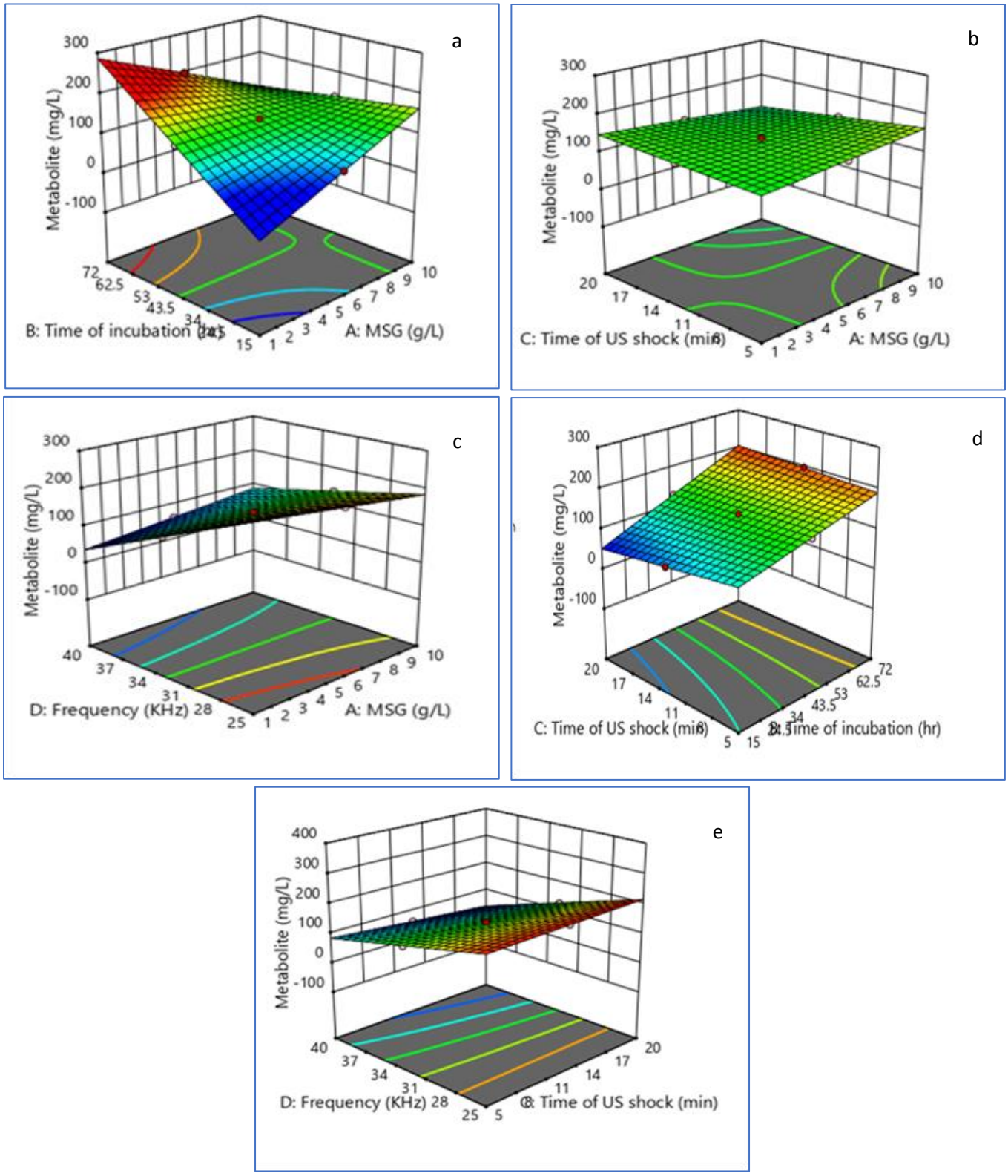

Fig. 1. Three-dimensional analysis of GABA aminobutyric acid production variables under the influence of ultrasonic shock in *L. brevis.*

hours of fermentation (21). GABA production begins in the bacterial growth phase and increases near the stationary phase due to increased GAD enzyme activity. An increase in glutamate enhances biomass production, but an excessive increase in glutamate is unfavorable and harmful for GABA production due to cell growth limitation during the rise in osmotic pressure (22). Monosodium glutamate is converted to L-glutamic acid through hydrolysis, which some strains do not

completely consume. As a result, the amount of GABA produced through this hydrolysis decreases (23). Ultrasound has become an important factor in the production of biological compounds. This process has been able to increase productivity by disrupting and destroying the cell wall. In this regard, low-frequency, low-energy ultrasound waves are crucial because this mechanism relies on the mass transfer from inside to outside of the cell (24). On the other hand, in this mechanism, cell rupture or porosity occurs when ultrasound waves hit the cell wall, resulting in a displacement inside and outside the cell, leading to GABA production at low frequencies (12). Gholamhosseinpour and Hashemi (25), in their investigation of ultrasound waves on milk fermentation containing *Lactobacillus plantarum* AF1 probiotic bacteria, stated that exposure to 30 kHz ultrasound for 5, 10 and 15 minutes resulted in the highest product yield in isoflavone production, milk antioxidant activity, and cell growth, which was consistent with the results of this research.

5. Conclusion

This study aimed to investigate the impact of ultrasound on GABA production by *Lactobacillus brevis* IBRC10818. The highest GABA production was achieved under the following conditions: 2.98% monosodium glutamate, 70.8 hours of incubation time, a shock time of 6.30 minutes, and a frequency of 33 kHz, resulting in a GABA yield of 212.2 mg/L. These optimal conditions were replicated three times, and the actual production amount was measured as 203.5 mg/L, showing consistency with the predicted values. The application of ultrasound shock has demonstrated its potential in enhancing the production of the GABA metabolite. The Design Expert software exhibited reliable performance in predicting the optimal production conditions. The utilization of lactic acid bacteria, often recognized as probiotics, for the bio-production of GABA, offers significant advantages in manufacturing Generally Recognized as Safe (GRAS) products for human consumption. Further studies are recommended to further explore additional variables, such as different carbon and nitrogen sources and various incubation temperatures, combined with ultrasound shock, to enhance the production of this valuable biological product.

Acknowledgment

We would like to express our gratitude to the staff and experts at the Biotechnology Laboratory of the Pharmaceutical Research Center, Faculty of Pharmacy, Shiraz University of Medical Sciences, for their support and assistance throughout this research.

References

1. Park KB, Oh S-H. Isolation and characterization of *Lactobacillus buchneri* strains with high γ-aminobutyric acid producing capacity from naturally aged cheese. *Food Science and Biotechnology*. 2006;15(1):86-90.
2. Chen MJ, Tang HY, Chiang ML. Effects of heat, cold, acid and bile salt adaptations on the stress tolerance and protein expression of kefir-isolated probiotic *Lactobacillus kefiranofaciens* M1. *Food Microbiology*. 2017;66:20-7.
3. de la Fuente B, Luz C, Puchol C, Meca G, Barba FJ. Evaluation of fermentation assisted by *Lactobacillus brevis* POM, and *Lactobacillus plantarum* (TR-7, TR-71, TR-14) on antioxidant compounds and organic acids of an orange juice-milk based beverage. *Food Chemistry*. 2021;343:128414.
4. Zhu W, Lyu F, Naumovski N, Ajlouni S, Ranadheera CS. Functional efficacy of probiotic *Lactobacillus sanfranciscensis* in apple, orange and tomato juices with special reference to storage stability and in vitro gastrointestinal survival. *Beverages*. 2020;6(1):13.
5. Dawan J, Ahn J. Bacterial stress responses as potential targets in overcoming antibiotic resistance. *Microorganisms*. 2022;10(7):1385.
6. Zhou H, Rao Z, Zhang Z, Zhou J. Function of the GABAergic system in diabetic encephalopathy. *Cellular and Molecular Neurobiology*. 2023;43(2):605-19.
7. Patil SB, Khan M. Germinated brown rice as a value added rice product: A review. *Journal of Food Science and Technology*. 2011;48(6):661-7.
8. Sun T, Zhao S, Wang H, Cai C, Chen Y, Zhang H. ACE-inhibitory activity and gamma-aminobutyric acid content of fermented skim milk by *Lactobacillus helveticus* isolated from *Xinjiang koumiss* in China. *European Food Research and Technology*. 2009;228(4):607-12.
9. Tujioka K, Ohsumi M, Horie K, Mujo K, Hayase K, Yokogoshi H. Dietary γ-aminobutyric acid affects the brain protein synthesis rate in ovariectomized female rats. *Journal of Nutritional Science and Vitaminology*. 2009;55(1):75-80.
10. Diana M, Quílez J, Rafecas M. Gamma-aminobutyric acid as a bioactive compound in foods: a review. *Journal of Functional Foods*. 2014;10:407-20.
11. Chew BL, Seymour GB. The effects of glutamate decarboxylase (GAD) RNAi knockout in tissue cultured transgenic tomato (*Solanum lycopersicum*). *Plant Omics*. 2013;6(1):13-7.
12. Hashemi SMB, Mousavi Khaneghah A, Saraiva JA, Jambrak AR, Barba FJ, Mota MJ. Effect of ultrasound on lactic acid production by Lactobacillus strains in date (*Phoenix dactylifera* var. Kabkab) syrup. *Applied Microbiology and Biotechnology*. 2018;102(6):2635-44.
13. Lejars M, Hajnsdorf E. RNase III participates in the adaptation to temperature shock and oxidative stress in *Escherichia coli*. *Microorganisms*. 2022;10(4):699.
14. Lee B-J, Kim J-S, Kang YM, Lim J-H, Kim Y-M, Lee M-S, et al. Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. *Food Chemistry*. 2010;122(1):271-6.
15. Behzadnia A, Moosavi-Nasab M, Tiwari BK. Stimulation of biosurfactant production by *Lactobacillus plantarum* using ultrasound. *Ultrasonics Sonochemistry*. 2019;59:104724.
16. Le PH, Verscheure L, Le TT, Verheust Y, Raes K. Implementation of HPLC analysis for γ-aminobutyric acid (GABA) in fermented food matrices. *Food Analytical Methods*. 2020;13(5):1190-201.
17. Rashmi D, Zanan R, John S, Khandagale K, Nadaf A. γ-aminobutyric acid (GABA): biosynthesis, role, commercial production, and applications. *Studies in Natural Products Chemistry*. 2018;57:413-52.
18. Zarei F, Nateghi L, Eshaghi MR, Abadi ME. Production of Gamma-aminobutyric acid (GABA) in whey protein drink during fermentation by *Lactobacillus plantarum*. *Journal of Microbiology, Biotechnology and Food Sciences*. 2020;9(6):1087-92.
19. Di Cagno R, Mazzacane F, Rizzello CG, De Angelis M, Giuliani G, Meloni M, De Servi B, Gobbetti M. Synthesis of γ-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: functional grape must beverage and dermatological applications. *Applied Microbiology and Biotechnology*. 2010;86:731-41.
20. Higuchi T, Hayashi H, Abe K. Exchange of glutamate and gamma-aminobutyrate in a Lactobacillus strain. *Journal of Bacteriology*. 1997;179(10):3362-4.
21. Komatsuzaki N, Nakamura T, Kimura T, Shima J. Characterization of glutamate decarboxylase from a high γ-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. *Bioscience, Biotechnology, and Biochemistry*. 2008;72(2):278-85.
22. Ranjan A, Singh S, Malani RS, Moholkar VS. Ultrasound-assisted bioalcohol synthesis: review and analysis. *RSC Advances*. 2016;6(70):65541-62.
23. Hong D-K, Jung S-E, Lee M-H, Lee H-J, Lee J-H, Na G-N, et al. Shelf-

life extension and increase in survivability of probiotics powder by ultrasonic treatment. *Journal of Milk Science and Biotechnology*. 2018;36(4):220-5.
24. Cho S-K, Jeong M-W, Choi Y-K, Shin J, Shin SG. Effects of low-strength ultrasonication on dark fermentative hydrogen production: Start-up performance and microbial community analysis. *Applied Energy*. 2018;219:34-41.
25. Gholamhosseinpour A, Hashemi SMB. Ultrasound pretreatment of fermented milk containing probiotic *Lactobacillus plantarum* AF1: Carbohydrate metabolism and antioxidant activity. *Journal of Food Processing and Engineering*. 2019;42(1).