PROCEEDINGS BOOK OF 11th ICEEE–2020 CONFERENCE
Indexed International Annual 11th ICEEE–2020 Conference
Sustainable Environmental Protection & Waste Management Responsibility
*Theme: "Sustainable Environmental Protection & Waste Management Responsibility"*
November 19 – 20, 2020 RKK – Óbuda University, Budapest, Hungary
ISBN: 978-963-449-203-0 www.iceee.hu

# EXTRACTION AND IDENTIFICATION OF PROBIOTIC BACTERIA WITH POTENTIAL OF GABA PRODUCTION FROM TRADITIONAL WEST AZARBAIJAN DAIRY OF IRAN

**Fatemeh Zarei[1], Leila Nateghi[2*], Hosam E.A.F. Bayoumi Hamuda[3], Maryam Zarei[4]**

*[1]Halal Research Center Islamic Republic of Tehran, Iran, Phone: +98-9129569137, Email: zarei.fatemeh@gmail.com,*
*[*2]Department of Food Science and Technology, Faculty of Agriculture, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran, Phone: +98-9125878775, Email: l.nathegi@iauvaramin.ac.ir,*
*[3]Institute of Environmental Engineering and Natural Sciences, Rejtő Sándor Faculty of Light Industry and Environmental Engineering, Óbuda University, Budapest, Hungary, Phone: +36303900813, E-mail: Bayoumi.hosam@uni-obuda.hu,*
*[4]community Nutrition Department, Ministry of health and medical education, Tehran, Iran, Phone: +98-9124168476, maryam.zarei@yahoo.com*

***Abstract:*** *Lactic acid bacteria play a critical role in fermentation courses. One of the metabolites produced by this bacterial strain is Gamma Amino Butyric Acid (GABA). The objective of this research was extraction and identification of probiotic bacteria with potential of GABA production from traditional West Azarbaijan dairy of Iran and optimization of GABA production conditions in culture. In this study, 30 bacterial samples from indigenous dairy products (yogurt, dough, cheese and butter) were isolated from West Azarbaijan of Iran. Initial diagnostic tests including gram stain, oxidase and catalase tests were performed to detect Lactic Acid Bacteria (LAB). Results showed that only 6 strains were gram-positive, negative catalase and negative oxidase, and were known as LAB. Then, investigation of probiotic properties including acid resistance, bile, gastric juice, and hemolysis inactivity and L-arginine hydrolysis were evaluated. Two LAB samples with strong probiotic properties were selected, followed by identification of the primers with 16SrDNA by sequencing and drawing phylogeny tree. The results showed that L. lactis subspecies Lactis, L. delbrucius subspecies Bulgaricus strains isolated from cheese and butter had the highest probiotic properties. In the second part, the concentration of GABA production by probiotic bacteria was evaluated by HPLC in media of MRS Broth. Based on HPLC results, L. delbrucius subspecies Bulgaricus, grown in MRS broth had shown the highest concentration of GABA production with 377.54 ppm.*

***Keywords:*** *Gamma aminobutyric acid (GABA), HPLC, L. delbrucius subspecies Bulgaricus, Probiotic, PCR*

## INTRODUCTION

Probiotics are viable and non-pathogenic microorganisms found in some foods that have a positive effect on

the host's health when taken in sufficient quantities. Ideal probiotics are part of the microflora, are involved in regulating immune responses, are capable of colonizing the human gastrointestinal tract, and inhibit the pathogenic bacteria from attaching to the intestinal mucosa [1]. It also has ideal properties such as resistance to gastric acid, bile salts, digestive enzymes and processing stages, ability to bind to intestinal epithelial cells, non-pathogenic and non-invasive, ability to maintain and maintain genetic stability, ability to cope with pathogens. Improve digestion of proteins and fats, vitamins synthesis, detoxification and protection of toxins [2]. Probiotics maintain and restore intestinal homeostasis, which can lead to several diseases, such as diarrhea, inflammatory bowel disease, irritable bowel syndrome, gastric ulcer, and even cancer [3]. Probiotics can be effective if they are resistant to passage through the stomach and intestine and do not disappear [4].

Many species of lactic acid bacteria (LAB) are generally known to be safe and several species of LAB have been recognized as safe by European food safety authorities [6]. Probiotics must be able to grow in the stomach and intestine and have the ability to attach to the intestinal wall and reside there in order to have beneficial effects on the body. For this purpose, there must be resistance to gastric acid chloride and bile salts present in the small intestine [7]. A minimum level of viable microorganisms is required to see a positive effect on their health; this level is usually between $10^8$ and $10^{11}$ colony forming unit (cfu), depending on the strains used and the health impact required [8]. Therefore, assuming daily consumption of 100 grams of dairy products, they should have $10^6$ to $10^9$ cfu/g of live bacteria at the time of consumption.

In general, LAB with probiotic properties are mainly used in various fermented foods, particularly dairy products. Based on their ability to synthesize gamma-aminobutyric acid (GABA), screening of LAB could open new prospects to GABA enriched dairy products [9]. GABA is a non-protein amino acid (AA) with the chemical formula of C4H9NO2 and a molecular weight of 103.12 g mol-1. This case is created by the activity of glutamate decarboxylase (GAD) in mitochondria through the irreversible decarboxylation of L-glutamate in the presence of pyridoxal-5'- phosphate coenzyme [10].

The biological roles of GABA include lowering of blood pressure in humans, diuretic effects, sleep regulation, insomnia and depression mitigation, and auto-immune response suppression, treatment of chronic alcohol-related illnesses, reduction of stress and stimulation of immune cells [11]. Hence, much attention is paid to GABA as a useful bioactive agent with potential healing properties in foods and pharmaceuticals [12]. Natural GABA was first recognized in potatoes and found in small quantities in several agricultural products such as barley, corn, cereals, fruits and vegetables including spinach, broccoli, tomatoes, apples and grapes [13]. In developed countries, GABA is used as a health AA. In addition, it is common as an extra supplement in numerous foods and nonprescription drugs used for many symptoms such as sleep disorders and stress. Relatively, studies on use of GABA supplementation in healthy individuals for up to 18 g for 4 days or 120 mg for 12 months have shown positive results [14]. Currently, use of functional foods containing GABA is increasing worldwide due to the important health benefits [15]. To the best of the author's knowledge, extraction and identification of probiotic bacteria with potential of GABA production from traditional West Azarbaijan dairy (Iran).

# MATERIALS AND METHODS

## Materials

Lactic acid bacteria were isolated from 30 samples of local yogurt, butter, dough and cheese purchased from the local market in West Azarbaijan of Iran. The culture media used were MRS Agar, Blood Agar, L-Arginine Amino Acid, purchased from Merck Co., Germany, as well as degenerate primers R 630, V 616 prepared by Takapoo Bios, Iran. Bile oxalate bile salts, hydrochloric acid, pepsin, trypsin, peptone juice, NaOH reagent were purchased from Merck Co., Germany. MRS broth was purchased from Sigma-Aldrich (USA). Triethylamine, acetonitrile, dihydrogen phosphate, acetonitrile and methanol were purchased from Merck (Germany), and phenyl isothiocyanate, orthophthalic aldehyde (OPA) and GABA standards were supplied by Sigma Aldrich (USA).

## Preparation of culture media and probiotic bacterial inoculation

To increase the number of microorganisms and ease of separation, 5 mg of each sample was transferred to MRS Broth medium. Samples were incubated in the incubator at 50 rpm for 1 week at 37°C to grow the isolated bacteria. During 1-week incubation, if needed, discarded culture medium was discarded and fresh media added again.

After the enrichment period, the microorganisms were isolated by centrifugation at 6000 rpm and suspended in 2 mM volume from the same culture medium. Serial dilution was then prepared in buffer solution of PBS phosphate salt solution from the above suspensions and cultured in the same medium. After 48 h incubation, the isolates were cultured on a new plate according to morphological characteristics. To maintain the isolated strains, the isolated microorganisms were cultured in the environment and under appropriate conditions and 25% sterile glycerol was added to them and stored in a freezer at -70°C until tests.

## Initial diagnostic tests

In order to identify lactic acid bacteria, initial diagnostic tests (Gram staining, catalase and oxidase) were performed on 30 samples of bacteria isolated from native dairy products (yogurt, butter, cheese and Dough) in West Azarbaijan of Iran [16].

## Evaluation of Probiotic Properties

The tolerance of the LAB isolates to both acidic pH value and bile salts, Arginine hydrolysis test, Hemolytic Activity and pepsin and trypsin test was studied using the methodology described [17].

## Molecular identification of selected strains

After performing the probiotic properties tests, the bacteria with the highest probiotic properties were selected for identification by 16SrDNA primers.

## Inoculation of probiotics in culture media

One colony of each *L. lactis subspecies Lactis, L. delbrucius subspecies Bulgaricus* was added to MRS broth incubated at 37°C for 24-48 h. To calculate number of bacteria inoculated in each milliliter of the suspension, 0.5 of McFarland standard was used. The optical density (OD) was measured at 625 nm with results in a range of 0.08-0.13 [18].

## Measurement of GABA using high performance liquid chromatography (HPLC)

Produced GABA in MRS broth was measured using reverse phase liquid chromatography. Derivation was conducted according to an original protocol by [19]. After centrifuging of media at 12000 ×g at 25°C for 10 min, 20 µl of the supernatant were poured into a 2-ml vial and then mixed vigorously with 20 µ1 of borate buffer. Then, 10 µl of OPA were added to the mixture stored at room temperature for 1 min. Then, 5 µl of 5% acetic acid were added to the mixture.

After derivation, 20 µ1 of each sample were injected to a capillary C18 Column, Rstech Hector-M (150 mm × 4.6 mm × 0.5 µm) at 25°C with UV-Vis detector (Younglin Acme 9000m, YL Instruments Co, South Korea) set at λ = 338 nm, 40 mM of sodium dihydrogen phosphate as mobile phase A (pH 7.8) and cetonitrile:methanol:water (10:45:45) as mobile phase B.

A stock solution of GABA (1 mg $ml^{-1}$) was prepared in water and diluted to 50% v v-1to obtain various concentrations. The analysis was carried out based on the corresponding calibration curves. Concentration of GABA was calculated by the comparison of the peak area with the corresponding GABA standard. The GABA concentration was reported as ppm.

## STATISTICAL ANALYSIS

The results of probiotic properties evaluation were analysed by Duncan's multiple range test at 95% confidence level using US version 16 software. Molecular identification results were performed using prism statistical software for selected Bacillus and sequencing for 16SrDNA sequence of Bacillus strains was performed using Blastn bioinformatics software and phylogenetic tree drawing was performed using MEGA version 4 of France was done.

All experiments were carried out in triplicate. The means comparison was carried out using Duncan's one way analysis of variance at a 95% confidence level. To optimize the production of GABA, RSM was used (Minitab Software v.16).

## RESULTS AND DISCUSSION

Identification of bacteria isolated from native dairy products in West Azarbaijan of Iran. The results of the initial diagnostic tests (gram stain test, catalase and oxidase) performed to identify lactic acid bacteria on 30 samples isolated from yogurt, buttermilk, butter and cheese are presented in Tables (1a and 1b).
The results showed that only 6 strains isolated from yogurt, buttermilk, Dough and cheese were gram positive, catalase negative and oxidase negative, and were confirmed as lactic acid bacteria and selected for further studies.

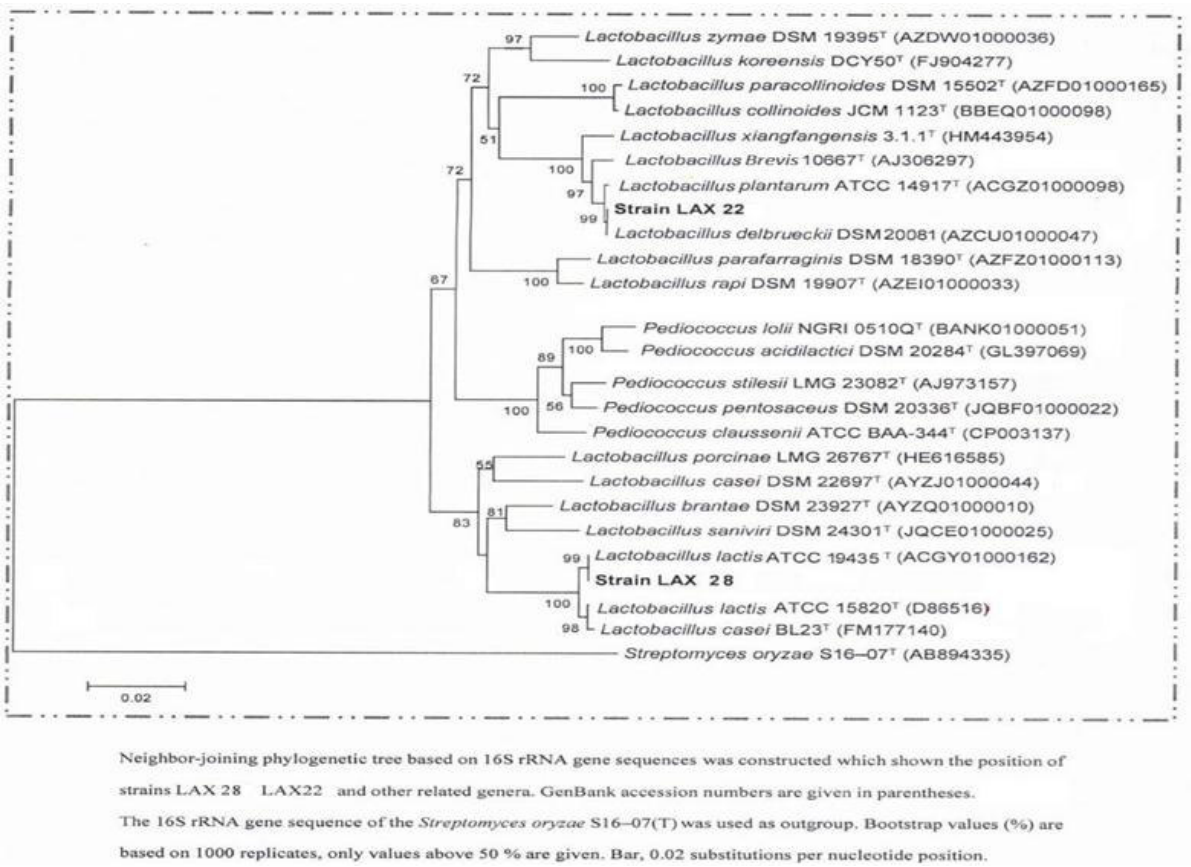
Table (1a):Results of Identification of bacteria isolated from native dairy products in West Azarbaijan of Iran.

| Treatment | Code | Source | Oxidase | Gram test | Catalase test |
|---|---|---|---|---|---|
| 1 | LAX-28 | Butter | Negative | Positive | Negative |
| 2 | LAX-148 | Cheese | Negative | Positive | Negative |
| 3 | LAX-22 | Yogurt | Negative | Positive | Negative |
| 4 | LAX-239 | Dough | Negative | Positive | Negative |
| 5 | LAX-239 | Cheese | Negative | Positive | Negative |
| 6 | LAX-239 | Yogurt | Negative | Positive | Negative |

Table (1b):Results of Identification of bacteria isolated from native dairy products in West Azarbaijan of Iran.

| Treatment | Code | Bile salts resistance | Acid resistance pH 2.5 | Trypsin resistance | Pepsin resistance | Hemolytic activity | L-Arginine |
|---|---|---|---|---|---|---|---|
| 1 | LAX-28 | 0.123±0.011e | 7.953±0.56b | 7.77±0.13a | 7.27±0.14a | Negative | Negative |
| 2 | LAX-148 | 0.337±0.019b | 6.263±0.24c | 5.393±0.19d | 6.25±0.21c | Negative | Negative |
| 3 | LAX-22 | 0.153±0.022d | 7.023±0.045a | 7.674±0.35b | 7.12±0.23ab | Negative | Negative |
| 4 | LAX-239 | 0.207±0.005f | 6.313±0.38b | 7.305±0.11b | 7.02±0.22b | Negative | Negative |
| 5 | LAX-239 | 0.208±0.013 c | 6.123±0.16d | 5.206±0.23d | 6.02±0.13d | Negative | Negative |
| 6 | LAX-239 | 0.408±0.028 a | 6.114±0.13d | 6.206±0.16c | 6.12±0.17cd | Negative | Negative |

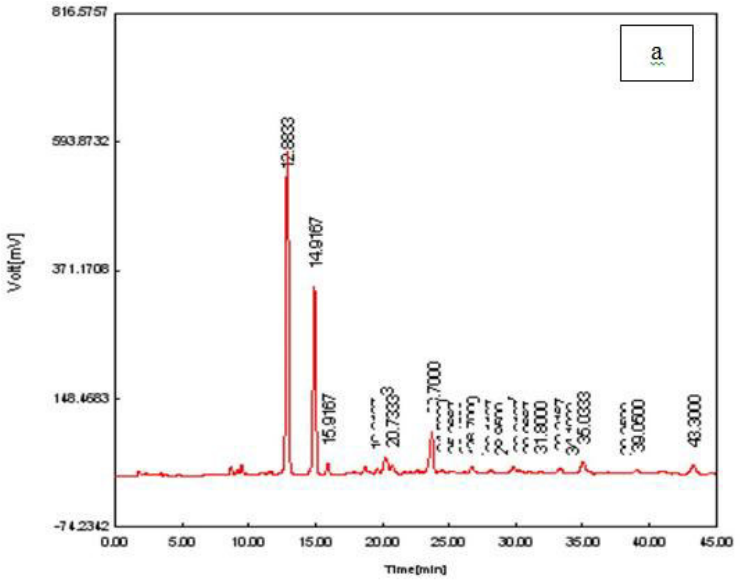
The results of Tables (1a and 1b) showed that only the LAX-28, LAX-22 strains isolated from yogurt and butter, respectively, were gram positive, catalase negative and oxidase negative and were confirmed as lactic acid bacteria and for further studies were chosen.
Probiotics are living microorganisms that, by being in the intestinal environment, can modify the microbial balance to increase their usefulness, and by their activity prevent the activity of non-beneficial and pathogenic microorganisms. All strains that have potential probiotic potential in order to be able to provide positive effects on the host are expected to tolerate gastrointestinal conditions.
The ability to tolerate acid, bile salts, and resistance to gastric juice are considered as good indicators for the survival of the strains in the gastrointestinal tract. These traits are usually evaluated first for selection of probiotic strains under culture conditions.
Although not all real-life conditions in the gut ecosystem can be provided under the conditions of the culture medium, it is still a powerful method for rapid screening of high-potential strains, allowing extensive study and study of a large number of strains. Enables probiotics to discover specific selection of probiotic strains under culture conditions, considering appropriate criteria, could isolate strains with more effective digestive capacity [20].
The molecular identification results of probiotic isolates are shown in Figure (1). All 2 strains were harvested using the 16SrDNA polymerase chain reaction of the D 616, V 616 degenerate primers prepared by Tekapoo Bios, whose sequence is as follows.




Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences was constructed which shown the position of strains LAX 28 LAX22 and other related genera. GenBank accession numbers are given in parentheses. The 16S rRNA gene sequence of the *Streptomyces oryzae* S16–07(T) was used as outgroup. Bootstrap values (%) are based on 1000 replicates, only values above 50 % are given. Bar, 0.02 substitutions per nucleotide position.

*Figure (1) Phylogenetic tree plotted in the SrDNA16 gene sequence*

According to the BLAST results, all samples were stratified by species similarity between 99-98%. Sequencing of the 16SrDNA region revealed that the selected strains showed the most affinities to the LAX-22, LAX -28 strains, respectively, as *L. lactis subspecies Lactis, L. delbrucius subspecies Bulgaricus strains.* Briefly, GABA was produced under the following conditions of temperature at 30°C for 42 h at pH 4 by *L. lactis subspecies Lactis, L. delbrucius subspecies Bulgaricus strains* in MRS broth (Table 2). Chromatograms are shown in Figures (2a and 2b). Of these bacteria, *L. delbrucius subspecies Bulgaricus* produced the highest GABA content of 377.54 ppm.



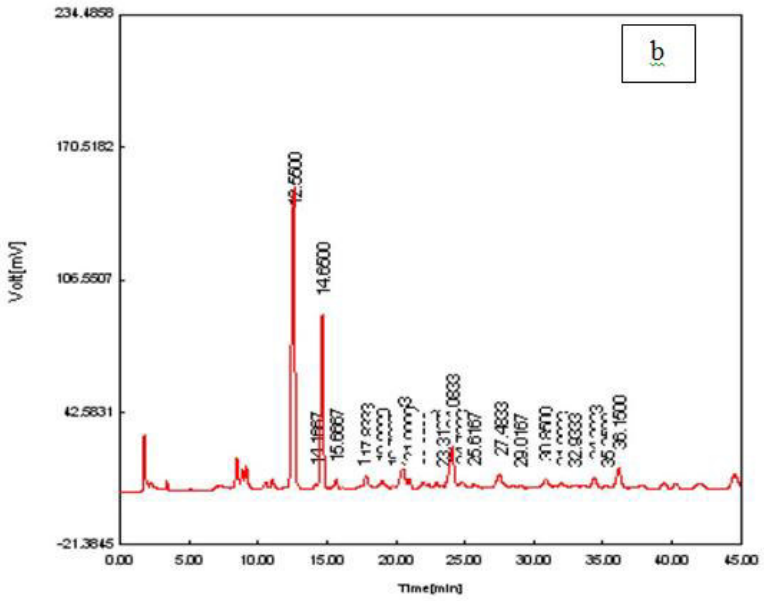



*Figure (2) Chromatograms of the gamma-aminobutyric acid (GABA) production by lactic acid bacteria in MRS Broth Figure (2a) L. lactis subspecies Lactis, (Figure b) L. delbrucius subspecies Bulgaricus strains*

Table (2) Gamma-amino butyric acid production in MRS broth (ppm)

| Media culture | Bacteria | GABA (ppm) |
|---|---|---|
| MRS | *L. lactis subspecies Lactis* | $301.09 \pm 10.99^{AB}$ |
| | *L. delbrucius subspecies Bulgaricus* | $377.54 \pm 15.12^{A}$ |

Different letters in each column show significant differences ($P \leq 0.05$)

*Lactobacillus plantarum* NDC75017 is a strain that is screened in traditional fermented dairy products in China. The inducing factors for GABA production included L monosodium glutamate (MSG) at 80 Mm, pyridoxal-5- phosphate as coenzyme of GAD at 18 µM and a culture temperature of 36°C. Under these conditions, activity of GAD in yogurt resulted in the production of 314.56 mg GABA per 100 g of the product [21]. A number of species and subspecies of lactic acid bacteria that produce GABA like: *Lactococcus lactis, Lactobacillus paracasea, Lactobacillus delbruci, Lactobacillus bosneri, Lactobacillus plantarum, Lactobacillus holoticus.* Among the lactic acid bacteria, *Lactobacillus* produces more GABA. Among the *Lactobacillus*, *Lactobacillus brevis* produce more GABA. All types of bacteria are separated from traditional foods such as kimchi, cheese, sourdough and paukai and so on. GABA-producing sources are the highest level of glutamate *Lactobacillus buchneri* isolated from kimchi produced GABA at a concentration of 251 mM was reported [22]. The highest content of GABA was found in Pecorino di Filiano (391 mg/kg). Among 22 different varieties of Italian cheeses, the responsible microorganisms for the GABA production were *Lb. paracasei* PF6, PF8 and PF13, *Lb. plantarum* PF14, *Lactobacillus* sp. strain PF7 and *Enterococcus durans* PF15 [23].

## CONCLUSION

In summary, the aims of the current study were assessment of potential GABA production by two probiotic bacteria extracted from **Traditional West Azarbaijan Dairy (Iran).** Of the two studied strains, *L. delbrucius subspecies Bulgaricus, grown in MRS broth had shown the highest concentration of GABA production with 377.54 ppm.*

## References

[1] Lars, A.: Lactic Acid Bacteria: Classification and Physiology. In, Hoang-Dung T., Fennema O., Hui Y., Walstra P., Karel M., et al. Lactic Acid Bacteria: Microbiological and Functional Aspects, 3th ed. New York, USA, Marcel Dekker Inc. 2004, p 18-85.

[2] Abdallyzadeh, M. Farahani Vasheghani, Ps. Khodabandeh, M. Hashemi Najaf–Abadi S.: Production of lactic

acid fermentation in non-continuous optimization process whey by Lactobacillus casei bacteria. J. Food Sci., 1389; 7(2): 95-102.
[3] El Soda M., Ahmed N., Omran N., Osman G. & Morsi A.: Isolation, identification and selection of lactic acid bacteria cultures for cheesemaking. *Emir. J. Agric.Sci.,* 2003; 15(2): 51-71.
[4] Yuliana N., Rangga A. & Rakhmiati E.: Manufacture of fermented coco milk-drink containing lactic acid bacteria cultures. *African J. Food Science*, 2010; 4(9): 558-562.
[5] Mehmood T., Masud T., Abbass A. & Maqsud Sh.: Isolation and Identification of Wild Strains of Lactic Acid Bacteria for Yoghurt Preparation from Indigenous Dahi. *Pakistan J. Nutrition*, 2009; 8(6): 886-871.
[6] Haddadin J.: Kinetic Studies and Sensorial Analysis of Lactic Acid Bacteria Isolated from White Cheese Made from Sheep Raw Milk. *Pakistan J. Nutrition*, 2005; 4(2): 78-84.
[7] Vasilean I., Segal R. & Vasile A.: Obtaining Fermented Dairy Products with The Yogurt Culture YF-L 812. The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology 2011; 35(1): 92-99.
[8] Tomas M., Ocana V., Wiese B. & Nader-Macarias M.: Growth and lactic acid production by vaginal Lactobacillus acidophilus CRL 1259, and inhibition of uropathogenic Escherichia coli. *J. Med Microbiol.,* 2003; 52(12): 1117-1124.
[9] Lengkey H. & Adriani L.: Effects of milk fermented with lactobacillus acidophilus and Bifidobacterium spp on lactic acid and acetic acid content and on Staphylococcus aureus and Pseudomonas aeruginosa. *Biotechnology in Animal Husbandry*, 2009; 25(5): 719-724
[10] Yamada K., Ota U. & Machinda H.: 1962. Studies on the Production of Lipase by Microorganisms. Part II: Quantitative Determination of Lipase. *Agricultural and Biological Chemistry*, 36(10): 860-864.
[11] Ayad E.H., Verheul A., Wouters J., & Smit G.: Population dynamics of lactococci from industrial, artisanal and nondairy origins in defined strain starters for Gouda-type cheese. *International Dairy Journal*, 2001; 11(1): 51-61.
[12] Hassaïne O., Zadi-Karam H., & Karam N.E.: Technologically important properties of lactic acid bacteria isolated from raw milk of three breeds of Algerian dromedary (Camelus dromedarius). *African Journal of Biotechnology*, 2007, 6(14):
[13] Ratanaburee A., Kantachote D., Charernjiratrakul W., Penjamras P. & Chaiyasut C.: Enhancement of γ-aminobutyric acid in a fermented red seaweed beverage by starter culture Lactobacillus plantarum DW12. *Electron J Biotechnol.*, 2011; 14(3): 1-14.
[14] Lim H.S., Cha I.T., Roh S.W., Shin H.H. & Seo M.J.: Enhanced production of gamma-aminobutyric acid by optimizing culture conditions of Lactobacillus brevis HYE1 Isolated from Kimchi, a Korean fermented food. *J Microbiol Biotechnol*., 2017; 27(3): 450-459.
[15] Park S.Y., Lee J.W. & Lim S.D.: The probiotic characteristics and GABA production of Lactobacillus plantarum K154 isolated from kimchi. *Food Sci Biotechnol*., 2014; 23(6): 1951-1957.
[16] Tserovska, L.; Stefanova, S. & Yordanova, T.: Identification of lactic acid bacteria isolated from Katyk, goat's milk and Cheese. *J. Cult. Collect*., 2002; 3: 48-52.
[17] Hassan Hassanzadazar, Ali Ehsani, Karim Mardani & Javad Hesari.: Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. *Veterinary Research Forum,* 2012; 3(3): 181-185.
[18] Lahuerta Zamora l. & Perez-Gracia M.T.: Using digital photography to implement the McFarland method. *J R Soc Interface*, 2012; 9: 1892-1897.
[19] Zarei F., Nateghi L., Eshaghi M. & Taj abadi M.: Optimization of GammaAminobutyric Acid Production in Probiotics Extracted from Iranian Dairy Products using MRS and Whey Protein Media. *Appl. Food. Biotechnol.,* 2018; 5(4):233-242.
[20] Dunne C., O'Mahony L., Murphy L., Thornton G., Morrissey D., O'Halloran S., Feeney M., Flynn S., Fitzgerald G., Daly C. & Kiely B.: In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *The American Journal of Clinical Nutrition*, 2001; 73(2): 386s-92s.
[21] Shan Y., Man C.X., Han X., Li L., Guo Y., Deng Y., Li T., Zhang L.W. & Jiang Y.J.: Evaluation of improved γ-aminobutyric acid production in yogurt using Lactobacillus plantarum NDC75017. *J. Dairy Sci.,* 2015; 98(4): 2138-2149.
[22] Cho Y.R., Chang J.Y. & Chang H.C.: Production of gamma aminobutyric acid (GABA) by Lactobacillus buchneri isolated from kimchi and its neuroprotective effect on neuronal cells. *J. Microbiol. Biotechnol.,* 2007; 17(1): 104-109.
[23] Siragusa S., De Angelis M., Di Cagno R., Rizzello C.G., Coda R. & Gobbetti M.: Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *J. Appl. Environ. Microbiol.,* 2007; 73(22): 7283-7290.