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Isolation and identification of probiotic bacteria with potential of Gamma Amino Butyric Acid production from traditional dairies in Iran

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Abstract

Background and objective: Lactic acid bacteria (LAB) is of interest in food fermentation studies. Gamma Amino Butyric Acid (GABA) is one of bioactive metabolites produced by LAB that pose significant impact on human health. At this study, we isolated the probiotic bacteria from Iranian traditional dairy products, identified them, and evaluated their potential in GABA production.

Materials and methods: In this research, 30 isolates of bacteria from traditional dairies (yogurt, dough, cheese and butter) were studied. To diagnose probiotic species, initial tests including gram staining, oxidase and catalase were performed. Additional verification studies on the approved isolated bacteria in the last step were included to acid, bile and pepsin and trypsin resistance, hemolysis inactivity and L-arginine hydrolysis. Probiotic bacteria were characterized by 16SrDNA PCR approach. In addition, GABA concentration was detected by HPLC equipped with C_{18} column (150 mm× 4.6 mm × 0.5 µm) at 25°C and UV-VIS detector (λ = 338 nm). The mobile phases of sodium dihydroxy phosphate and acetonitrile/ methanol/water were used in gradient mode.

Results and conclusion: Among all isolated bacteria, six strains were gram-positive, catalase-negative and oxidase-negative that initially known as LAB. Two out of six LAB isolates with acceptable probiotic characteristics were selected based on supplementary tests. Accordingly, *Lactobacillus lactis* ssp. *Lactis* and *Lactobacillus delbrueckii* ssp. *Bulgaricus* isolated from cheese and butter, respectively, showed the most significant probiotic properties. However, *Lactobacillus delbrueckii* ssp. *Bulgaricus* produced higher GABA than another (377.54 vs. 301.09 mg l^{-1}). As conclusion, traditional dairies in Iran are good reservoir of indigenous healthy microorganisms that can be used as starter or adjuvant culture with several helpful impacts on consumers.

Keywords: Gamma Amino Butyric Acid, HPLC, *L. delbrueckii* ssp. *Bulgaricus*, *L. lactis* ssp. *Lactis*, PCR, Probiotic

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1. Introduction

Probiotics are introduced as non-pathogenic and non-invasive biologic organisms found in some foods that have positive impact on host's health when ingested in sufficient quantities. They may be a part of normal microflora or orally administered through which are involved in regulating the immune responses and capable of colonizing in human gastrointestinal tract thereby inhibited the pathogen's attachment to the intestinal mucosa. These characteristics are facilitated by their ability to tolerate the stomach juice, bile salts and digestive enzymes. Moreover, they improve digestion of proteins and fats, synthesize vitamins and extend detoxification [1,2]. Indeed, at least 10^6 and 10^9 colony forming unit (CFU) of alive healthy microorganisms per day should be administered to achieve the expected results [3].

Some species of lactic acid bacteria (LAB) are generally recognized as safe by US Food and Drug Administration [4]. In general, probiotic LAB are extensively used in food biotechnology especially for preparation of dairy products through which they able to produce several bioactive ingredients [5]. Gamma Amino Butyric Acid (GABA) is a non-protein amino acid with chemical formula of $C_4H_9NO_2$ and molecular weight of 103.12 g mol^{-1} that is one of functional compounds produced by LAB. It is produced by glutamate decarboxylase in mitochondria through irreversible decarboxylation of L-glutamate in the presence of pyridoxal-5'-phosphate co-enzyme [6]. The favorable roles of GABA include antihypertension, causing diuresis, regulation of sleep, mitigation of insomnia and depression, suppression of auto-immune response, stimulation of immune cells, treatment of alcohol-associated chronic diseases, and stress suppression [7].

Currently, intake of functional foods containing GABA is increasing across the globe as a result of its approved health impacts [8]. Based on the ability of LAB to synthesize GABA, their screening could open new prospects to dairy industries [9]. The current study aimed to isolate LAB from traditional dairy products of West Azerbaijan in Iran and screening the most potent probiotic bacteria with the potential of GABA production.

2. Materials and methods

2.1. Materials

LAB were isolated from 30 samples of local yogurt, dough, cheese and butter purchased from local market in West Azerbaijan, Iran. L-Arginine amino acid and culture media of MRS broth/agar and blood agar were purchased from Merck (Germany). Degenerate primers of R 630 and V 616 were supplied by Takapoo Bios Company (Iran). Bile oxalate, bile salts, hydrochloric acid, pepsin, trypsin, peptone juice and NaOH were purchased from Merck Co., Germany. Acetonitrile, triethylamine, methanol, dihydrogen, phosphate, phenyl isothiocyanate, orthophthalic aldehyde (OPA) and GABA standard were purchased from Sigma Aldrich Company (USA).

2.2. Preparation of culture media and probiotic inoculation

To enrich the microorganisms, 5 mg of each sample was transferred to MRS broth o.3% (w v^{-1}). Inoculated samples were placed in shaker incubator at 37°C and 50 rpm for one week. Then, the microorganisms were isolated by centrifugation at 4025 ×g after discarding the supernatant and transferring the precipitate to 2 ml of MRS broth. Serial dilution was then prepared in MRS broth (incubation at 37°C for 48 h). The enriched cultures were plated on MRS agar (incubation at 37°C for 48 h) followed by verification tests based on morphological characteristics. The confirmed probiotics were stored under freezing at -70°C until analysis [10].

2.3. Initial diagnostic tests

In order to identify LAB, initial diagnostic tests (gram-staining, catalase and oxidase) were performed on 30 isolates of bacteria [10].

2.4. Evaluation of probiotic potency

Resistance to acidic pH, bile salts, pepsin and trypsin, arginine hydrolysis, and haemolytic activity were monitored according to the method presented by Hassanzadazar et al. [11].

2.5. Molecular identification of selected probiotics

Following to diagnostic tests, the bacteria with the highest probiotic potency were selected for identification by 16SrDNA PCR approach according to the method of kwon et al. [12]. Phylogenetic tree was prepared upon NCBI databases. 16SrDNA from several lactobacilli bacteria were sequenced and identified using ClastalW software [13].

2.6. Determination of GABA by high performance liquid chromatography

Concentration of GABA was calculated according to the method of Zarei et al. [14]. MRS broth, inoculated with *Lactobacillus delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis* at 30°C for 42 h, was centrifuged at 8064 ×g at 25°C for 10 min. Then, 20 µl of supernatant was transferred to 2-ml vial and 20 µl of borate buffer was added and mixed. Consequently, 10 µl of OPA was added to the mixture. After 1 min, 5 µl of acetic acid 5% (v v^{-1}) was added. After derivation, 20 µl of each specimen was injected into a C_{18} column (Dione, USA) (150 mm× 4.6 mm × 0.5 µm) at 25°C. Determination was done by UV-VIS detector at λ= 338 nm. Two mobile phases of A and B including 40 mM sodium dihydrogen phosphate (pH=7.8) and acetonitrile/methanol/water (10:45:45, v:v:v) were used. Isolation was done in feed flow rate of 1 ml min^{-1} in gradient mode of 45 min for each cycle. Standard curve was prepared by reading the absorbance of GABA standard solutions at concentrations of 50, 100, 200, 300, 400 mg l^{-1} at λ= 338 nm.

2.7. Statistical analysis

The means of three replicates were compared by one-way analysis of variance followed by Duncan's test at 95% confidence interval using Minitab software version 16. Phylogenetic tree was drawn by MEGA software version 4.

3. Results and discussion

3.1. Initial diagnostic tests on the probiotic bacteria isolated from traditional dairy products

The results of initial diagnostic tests on 30 isolated bacteria from yogurt, dough, cheese and butter are presented in Table 1. In total, six strains of LAX-28, LAX-148, LAX-22, LAX-239, LAX-230 and LAX-231 were gram-positive, catalase-negative and oxidase-negative and confirmed as LAB that were selected for further studies.

3.2. Probiotic potency of LAB isolated from traditional dairy products

Ability of microbial species to survive and be active under exposure to low pH, bile salts and digestive enzymes are of pre-defined indicators for classification of probiotics [15]. At first, the evaluations are done under simulated conditions that are comparable to human gastrointestinal tract in spite of some differences and restrictions. As mentioned by Hassanzadazar et al., simulated digestive tract can help in isolation of the most potent microorganisms for further clinical studies [11]. Our results of probiotic diagnostic tests under simulated gastrointestinal tract are shown in Table 2. The results showed all six strains were similar in hemolysis and L-arginine tests but LAX-28 and LAX-22 strains isolated from yogurt and butter, respectively, were more resistant to acid (pH=2.5), bile salts, pepsin and trypsin than the others and were selected for molecular identification. With regard, 88 species of lactobacillus were isolated from traditional unpasteurized milk and cheese in France, through which only four species showed the best probiotic characteristics [16].

Table 1- Results of initial identification of bacteria isolated from traditional dairy products (yogurt, dough, cheese and butter)

Treatment	Code	Source	Oxidase	Gram-staining	Catalase
1	LAX-28	Butter	-	+	-
2	LAX-148	Cheese	-	+	-
3	LAX-22	Yogurt	-	+	-
4	LAX-239	Dough	-	+	-
5	LAX-230	Cheese	-	+	-
6	LAX-231	Yogurt	-	+	-
7	LAX-183	Butter	+	-	+
8	STX-53	Cheese	+	-	+
9	LAC-4	Yogurt	+	-	+
10	DLP-2	Dough	+	-	+
11	TD3	Cheese	+	-	+
12	T2	Yogurt	+	-	+
13	LAR-7	Dough	+	-	+
14	LAA-5	Cheese	+	-	+
15	LAA-10	Yogurt	+	-	+
16	BIA-6	Yogurt	+	-	+
17	BIA7	Butter	+	-	+
18	T6	Cheese	+	-	+
19	T12-1	Yogurt	+	-	+
20	T15	Yogurt	+	-	+
21	T21-2	Dough	+	-	+
22	T23-2	Cheese	+	-	+
23	TD9	Yogurt	+	-	+
24	TD11	Yogurt	+	-	+
25	TD14	Cheese	+	-	+
26	TD15	Yogurt	+	-	+
27	LAX-279	Dough	+	-	+
28	LAX-148	Cheese	+	-	+
29	LAX-149	Yogurt	+	-	+
30	LAX-236	Yogurt	+	-	+

Table 2- Results of probiotic diagnostic tests on selected bacteria through initial identification

Treatment	Code	Acid resistance (pH=2.5)	Bile salts resistance	Pepsin resistance	Trypsin resistance	Hemolytic activity	L-arginine
1	LAX-28	7.953±0.56[a]	0.123±0.01[e]	7.27±0.14[a]	7.77±0.13[a]	-	-
2	LAX-148	6.263±0.24[b]	0.337±0.02[b]	6.25±0.21[c]	5.393±0.19[d]	-	-
3	LAX-22	7.023±0.04[a]	0.153±0.02[d]	7.12±0.23[ab]	7.674±0.35[b]	-	-
4	LAX-239	6.313±0.38[b]	0.207±0.00[f]	7.02±0.22[b]	7.305±0.11[b]	-	-
5	LAX-239	6.123±0.16[b]	0.208±0.01 [c]	6.02±0.13[d]	5.206±0.23[d]	-	-
6	LAX-239	6.114±0.13[b]	0.408±0.03 [a]	6.12±0.17[cd]	6.206±0.16[c]	-	-

*The numbers are log CFU.

3.3. Molecular identification of LAX-28 and LAX-22 strains isolated from traditional dairy products

Figure 1 shows the phylogenetic tree of selected probiotics. Two strains of LAX-22 and LAX-28 were harvested by 16SrDNA polymerase chain reaction using D 616 and V 616 degenerate primers. According to the BLAST results, all samples were classified based on species similarity of 99-98%. Sequencing of 16SrDNA region revealed that the selected strains of LAX-22 and LAX-28 corresponded to *L. delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis.*

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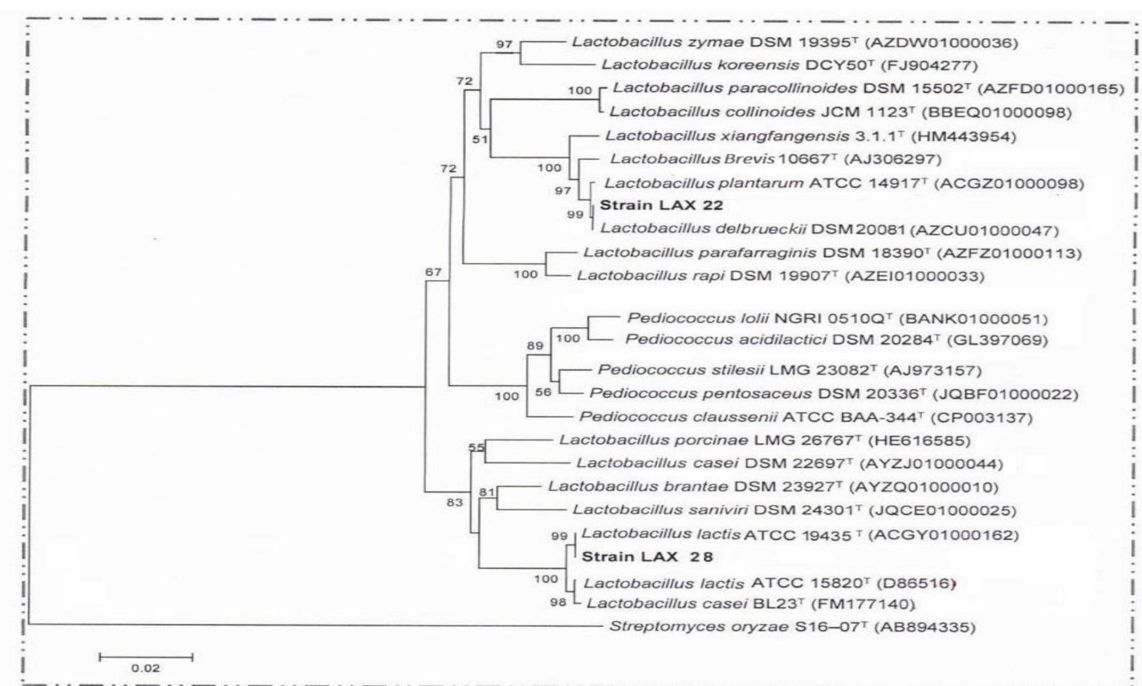

Figure 1. Phylogenetic tree of LAX-22 and LAX-28 strains according to 16SrDNA gene sequencing

3.5. GABA production by *L. delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis* isolated from traditional dairy products

LAB are of essential organisms in food fermentation processes and have a long history of safe use by human. One of favorable metabolites produced by these bacteria is GABA that is of great interest in food and pharmaceutical industries [17]. It was observed that GABA can improve the eyes function and memory in animals. In agreement, daily intake of wheat germ containing 26.4 mg of GABA could alleviate neurological diseases symptoms [18]. Several species of LAB can produce GABA; among them, Lactobacillus is the most potent [19]. The results of GABA production by *L. delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis* in our study are presented at Table 3 and Figure 2. Accordingly, *L. delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis* produced GABA at concentrations of 377.54 and 301.09 mg l^{-1}, respectively.

In comparison, the highest concentration of 391 mg kg^{-1} GABA was produced in Pecorino di Filiano Italian cheese by 22 isolates of *L. paracasei* PF6, PF8 and PF13, *L. plantarum* PF14, *Lactobacillus* spp. strain PF7 and *Enterococcus durans PF15* were studied by the Italian researchers [20].

L. plantarum NDC75017 was isolated from traditional fermented dairy products in China. Under optimum condition (80 Mm of L-monosodium glutamate and 18 μM of pyridoxal-5-phosphate at 36°C), it produced 314.56 mg GABA per 100 g of yogurt [21]. In addition, *L. buchneri* isolated from kimchi produced GABA at concentration of 13.05 mg l^{-1} [22]. Other scientists detected 23.40 mg l^{-1} of GABA in black raspberry juice produced by *L. brevis* [23]. Tajabadi et al. 2015 isolated 24 species of lactobacilli from honey in the stomach of bees that showed *L. plantarum Apis362 Taj* had the highest ability to produce GABA (7.15 mg ml^{-1}) [24].

Table 3- Gamma Amino Butyric Acid production in MRS broth (mg l^{-1}) by L. *delbrueckii ssp. Bulgaricus* and *L. lactis ssp. Lactis*

Bacteria	GABA (mg l^{-1})
L. lactis ssp. *Lactis*	301.09 ±10.99
L. delbrueckii ssp *Bulgaricus*	377.54 ±15.12

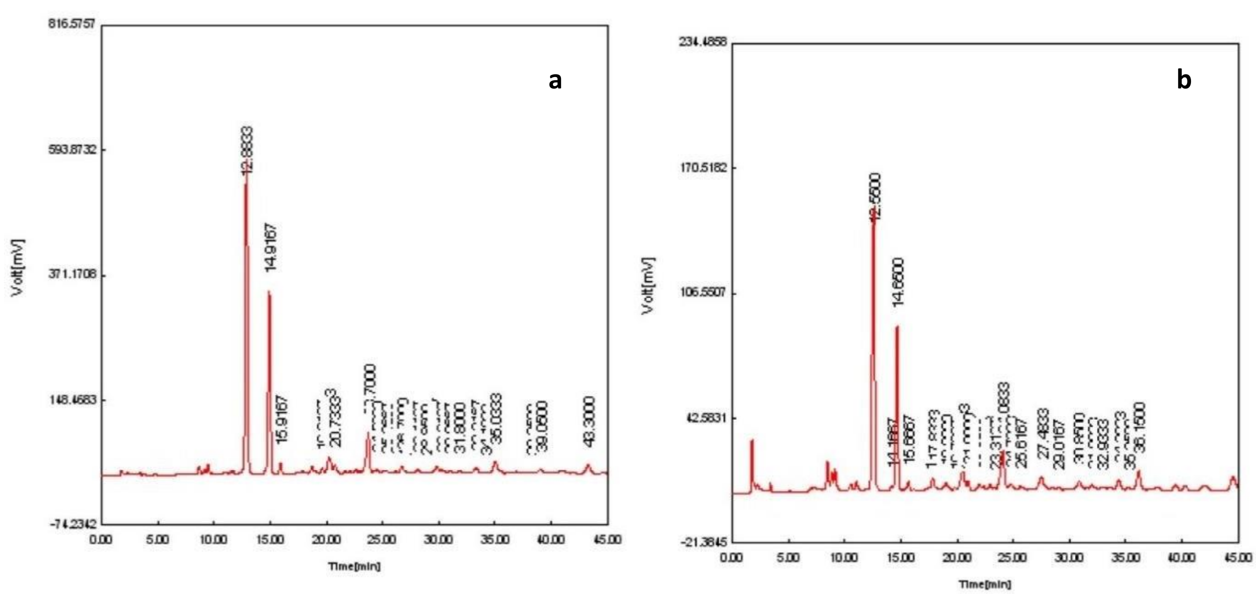

Figure 2- Chromatograms of Gamma Amino Butyric Acid production by a) *L. lactis* ssp. *Lactis*, b) *L. delbrueckii* ssp. *Bulgaricus*

4. Conclusion

At this study, six out of 30 bacterial isolates of traditional dairy products (yogurt, dough, cheese and butter) were detected as LAB. Furthermore, the two isolates that showed the highest probiotic attributes were *L. delbrueckii* ssp. *Bulgaricus* and *L. lactis* ssp. *Lactis* according to the phylogenetic tree. Relatively, GABA production at concentrations of 377.54 and 301.09 mg l^{-1} in MRS broth were detected by the species, respectively. We concluded that the isolated probiotics can be introduced as new healthy species able to produce GABA in foods. This achievement is useful in formulation of novel functional foods to help in prevention of some diseases. However, further studies are needed for optimization and in vivo evaluation.

5. Conflict of interest

The authors declare no conflict of interest.

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