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Production of Vitamins B_3, B_6 and B_9 by Lactobacillus Isolated from Traditional Yogurt Samples from 3 Cities in Iran, Winter 2016

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Abstract

Background and Objective: B-group vitamins have important roles in many aspects of cellular metabolism and humans cannot synthesize them. So, they should be obtained from external resources. This project provides a new insight into assessing the production of vitamins B_3, B_6 and B_9 by Lactobacillus, isolated from traditional yogurt samples from 3 different cities of Iran; Golpayegan, Sanandaj and Tehran (Damavand).

Material and Methods: Following 72 h of anaerobic culture of the Lactic acid bacteria at 37°C in 5% CO_2, some Lactobacillus species from traditional yogurt samples were isolated and characterized both morphologically and biochemically. Isolates were identified following 16S rRNA PCR-amplification and sequencing. Including *Lactobacillus (L.) ozensis* strain Gon2-7, *L. acidophilus* strain KU, *L. helveticus* strain D76, *L. helveticus* strain Dpc 4571, *L. fermentum* strain 1, *L. rossiae* strain DSM15814T, *L. casei* strain NCDO, *L. delbrueckii* strain ATCC 11842, *L. crispatus* strain MRS 54.4, *L. delbrueckii* strain SB3 and *L. paracasei* subsp. *tolerans* JCM1171 (T). The sequence of *L. paracasei* subsp. *tolerans* JCM1171 (T) was submitted to the NCBI. The ability to produce B-group vitamins was evaluated by high performance liquid chromatography. Lactobacillus strains and amount of vitamin B_3, B_6 and B_9 production were analyzed by Analysis of Variance test.

Results and Conclusion: Eleven isolates of Lactobacillus species from traditional yogurt samples were identified. Optimal conditions for Lactobacillus growth were pH 5-6 and temperatures 37-40°C. The isolates produced vitamins B_3, B_6 and B_9. *L. paracasei* subsp. *tolerance* JCM 1171 (T) showed the highest amount of produced vitamins ($p \leq 0.01$) consist of vitamin B_6 (1566.17 µg ml^{-1}) and B_9 (1279.72 µg ml^{-1}). *L. acidophilus* strain KU showed the highest production of vitamin B_3 (522.7 µg ml^{-1}). *L. fermentum* produced the highest amount of vitamin B_2. These strains are a natural and cost efficient source of vitamin. The Lactobacillus strains isolated in this research particularly, *L. paracasei*, could be applied in improving new fermented products, fortified with B-group vitamin that could be applied as substitution for enriching and supplementation with the controversial synthetic vitamins.

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1. Introduction

While most vitamins are available in different kind of foods, vitamin deficiency still happens in the world, due to unstable and unhealthy diets. Vitamins are essential micronutrients that humans must obtain daily through the diet [1].

Humans are unable of synthesizing B-group vitamins, and they accordingly have to be obtained from their diet. However, due to the unwanted side effects associated with excess intake and the high cost, research has shifted into using microorganisms in the production of vitamins as a cost-effective and consumer friendly alternative to their chemically synthesized counterparts [1,2].

The idea of using Lactic Acid Bacteria (LAB) as probiotic bacteria in dairy products is not new. A large

group of LAB belong to the genus Lactobacillus which has an extended history of safe use, particularly in dairy products. By producing nutrient compounds such as vitamins, these bacteria reduce the growth rate of harmful diseases caused by bacteria. Vitamin production caused by carbohydrate fermentation by lactobacilli is of great value in the food fermentation, medical, pharmaceutical, and chemical industry [3].

Probiotics are live microorganisms that provide health benefits when consumed [4]. Probiotics are used in both food and medicine, and they are capable of improving both food safety and human health in an endogenous and an exogenous manner [4,5].

Lactobacilli are capable of producing compounds such as bacteriocins, antibiotics and vitamins including those belonging to the B-group, all of which improve host health, protect against pathogens and improve the immune system [6-8]. Human life could not exist in the absence of B-group vitamins as these vitamins are an essential component in cellular metabolism, including DNA replication and repair as well as nucleotide, vitamins and certain amino acid biosynthesis [1,8,9]. B-group vitamins have the ability to solve in water and play an important role in the metabolism of carbohydrates, proteins and lipids. Members of vitamin B-group consist of Thiamin (B_1), Riboflavin (B_2), Niacin (B_3), Pantothenic acid (B_5), Pyridoxine (B_6), Biotin (B_7), folate (B_9) and Cobalamin (B_{12}) [10,11]. Vitamin B_{12} (Cobalamin) is one of the most important members of the B group vitamin which is a complex compound that is only naturally produced by few bacteria such as some kind of Lactobacillus and Propionibacterium. *Lactobacillus (L.) reuteri* and *L. roseiae* could improve B_{12} production by enriching of the medium. The biosynthesis of vitamin B_{12} is divided in three sections: 1: the synthesis of uroporphyrinogen III from succinyl-CoA and either glutamyl-tRNA or glycine, 2: synthesis of the corrin ring 3: Adenosylation of the corrin ring [12]. As over 70 steps are needed for chemical synthesis of vitamin B_{12}, some microorganisms can produce vitamin B_{12} by the fermentation process. Expression of 10 genes of the hem, cob (cob U and cob S) and cbi gene families required for vitamin B_{12} synthesis in *Propionibacterium* (*P.*) *freudenreichii*, which can increase the rate of Vitamin B_{12} production. Multi gene expression in *P. freudenreichii* is the main reason for the increased vitamin B_{12} production [13].

To contribute to this end, it was aimed to characterize the capability of B-group vitamins production by lactic acid bacteria isolated from several traditional yogurt samples prepared from different regions of Iran. The main aim for current study was to evaluate the production of vitamins B_3, B_6 and B_9 by Lactobacillus strains particularly *L. paracasei* subsp. tolerans JCM 1171 (T).

2. Materials and Methods

2.1. Yogurt Samples

Traditional Iranian yogurt samples from different regions (Golpayegan, Sanandaj, Tehran and Damavand) were collected in sterile covered, small, labeled containers and transferred to the Laboratory on ice.

2.2. Sample enrichment and standard microbiology tests

Sample enrichment was performed by re-suspending a volume of 1 mL of each sample in 9 mL of physiological serum. Serial dilutions of 1:1000, 1:10000 and 1:100000 were then prepared in physiological serum from which a 20 µl sample was sub-cultured in duplicate into the MRS broth medium containing flasks (MRS broth from Merck, Germany). Then, these flasks were incubated in 5% CO_2 for 72 h at 37°C. Following bacterial colony formation, lactic acid bacteria were initially identified according to standard bacteriological methods [11,14,15].

2.2.1. Gram staining test

Gram staining procedure was used to analyze the isolated bacteria for primary identification [14], and were detected by light microscope (KF2, Zeise Germany) with magnification of 1000 [13,14].

2.2.2 Catalase test

A single colony was isolated and then streaked on a glass slide and one drop of 3 % H_2O_2 (hydrogen peroxide) was added on it (Merck, Germany). The bubbling of oxygen showed the positive response [16,17].

2.2.3 Oxidase test

For the oxidase test, at first, filter paper discs in petri dishes were impregnated with N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride (1%), into which was transferred the bacterial colonies. There is no colour changes in oxidase negative [16,17].

2.2.4 TSI (Triple Sugar Iron) test

TSI test is based on the ability of bacteria to ferment the three carbohydrate molecules of glucose, lactose and sucrose and produce acid and hydrogen sulfide. The base medium for TSI test was Phenol red broth. The medium also contained agar, different sugar substrates including glucose (0.1% v v^{-1}), sucrose (1% v v^{-1}) and lactose (1% v v^{-1}) and ferrous sulfate and sodium thiosulfate, (Merk, Germany). To perform this test, a single colony of the bacteria were inoculated to each tube and incubated for 72 h at 37°C [18].

2.2.5 Motility test

A single colony of the bacteria were inoculated to the tubes of semi-solid SIM (Sulphide Indole Motility

medium) (Merk, Germany) and incubated for 72 h at 37°C [18]. For bacteria which were motile, the growth lines spread in the semisolid SIM medium [17,19].

2.2.6 Carbohydrate fermentation test

Within a chain of regular and specific enzymatic reactions conduction to the bio-oxidation of regularly a carbohydrate, most microorganisms catch their energy. Then, to distinguish the fermentation specification and subsequent characterization of Lactobacillus isolates different carbohydrate were used. Initially the basal medium was prepared (bioMerieux, Tokyo, Japan). Reagents used for the preparation of basal medium were, Yeast extract (8 g), Tween 80 (1 mL), Peptone (10 g), Di-potassium hydrogen phosphate (2 g), Di-ammonium hydrogen citrate (2 g), Sodium acetate (5 g), , Magnesium sulfate (0.2 g), Manganese (II) sulfate (0.4 g), double distilled H_2O to 1000 mL and distributed into separate tubes, to each of which was added the different sugar substrates namely, lactose, sucrose, galactose, arabinose, manitol, sorbitol, fructose, ramnose and ribose (Bio Maxima, Poland) at a concentration of 2% each. A single colony of the bacteria were inoculated to the tubes and incubated for 48 h at 37°C. The changes in the color of the medium showed positive response of the bacteria to this test [17,20].

2.2.7 Growth at different pH

Adjusting to different pH was done by NaOH (1.0 M) and HCl (1.0 M) to a single isolated colony which was sub-cultured in MRS broth and incubated at 37°C for 48-72 h to detect growth of lactobacilli under different pH values of 2, 3, 4, 5, 6, 9 and 10 using a spectrophotometer. The optical density values at 600nm were recorded [20,21].

***2*.2.8. Bile salt tolerance**

Bile salts tolerance of the isolates was defined matching to the technique explained by Menconi et al. [22]. Oxgal 0.3% (Merk, Germany) was added to MRS broth medium. A single colony of bacteria was inoculated to MRS broth tubes then incubated at 37°C for 48 h [21,22].

2.3. Molecular identification of Lactobacillus strains

A key for phylogenetic analysis and detection is sequence of nucleotide base for 16S rDNA of Lactobacillus [24]. The following steps were performed for the purpose of strain identification through 16S rDNA sequencing [22,23].

2.3.1. DNA Extraction

Extraction of DNA was performed from pure cultures prepared from bacterial colonies using the "Biospin Bacteria Genomic DNA Extraction" Kit (Bioneer, USA) and kept at -20°C.

2.3.2. rDNA gene PCR amplification

rDNA gene PCR amplification from bacterial genomic DNA samples was performed using lactobacilli-specific primers. For this purpose, the universal common primers, forward 27F (5-AGAGTTTGATCCTGGCTCAG-3) and reverse 1492R (5′-GGTTACCTTGTTACGACTT-3) were used for amplification of 16S rRNA with the PCR technique corresponding to the flanking terminal region of the 16S rRNA gene [24]. Mixture of the PCR reaction (25 µl) included 1.0 U Taq DNA polymerase (Amplicon-Denmark), primer mixture comprising of 20 pmol primer, PCR Master Mix and DNA template prepared as described above. A negative control sample (no DNA template) was also included for possible foreign DNA contamination (data not shown). Performance of PCR amplification was carried out with a Bioer thermal cycler (Ferrotec, Japan) and DNA fragments which were amplified as follows: 1) 94°C for 5 min for initial heating, 2) 94°C for 60 s for denaturation, 3) 56°C for 60 s for annealing, 4) 72°C for 60 s for extension, 5) 5 min at 72°C for final extension. The PCR product (5 µl) underwent gel electrophoresis (Syngene G: BOX, USA) on agarose (1% w w^{-1}) (Merck, Germany), followed by staining with ethidium bromide solution (Cinna colon, Iran) [23,24,25].

2.3.3. Sequence analysis of the 16S rRNA gene from isolated lactobacilli

The PCR products were sequenced by Macrogene Company (Korea). The chromatograms were edited using the Chromas version 3.1 software. BLAST analysis was performed at EZTaxon and NCBI GenBank databases. The phylogenetic tree was drawn according to molecular data with Mega 6 program. Bootstrap support values were calculated from 1.000 replicates.

2.4. HPLC analysis

The identified Lactobacillus strains were cultured in MRS broth medium for 72 h prior to a 10 min centrifugation at 10000 ×g. The supernatant was removed from the bacterial pellet and passed through a 0.25 µm syringe filter. The filtrate was then re-cultured for 72 h in MRS broth medium to make sure of its sterility (no Lactobacilli growth). Twenty microliters of the filtrate was injected into the HPLC system. Chromatographic separation was achieved on reversed-phase-HPLC columns HPLC columns C18 (25cm × 4.6 mm) through an aqueous mobile phase (phosphate buffer-CH3CN 10 mM, at a flow rate of 1 mL min^{-1}, pH 3.6, (UV) absorbance was recorded at 282 nm at room temperature at 25°C. Quantification of vitamin B_3, B_6 and B_9 content was performed on a Waters Alliance 2790 HPLC system [26,27,28]. Vitamin B-group standards with 99% purity produced by American Sigma Aldrich Company were used.

2.5. Statistical analysis

Assays were conducted in triplicates and statistical analysis was performed using SPSS ver. 18. The comparisons of differences between the means of the treatments were tested by one way Analysis of variance (ANOVA) and $p \leq 0.01$ was considered as significant.

3. Results and Discussion

3.1. Initial identification of lactobacilli

3.1.1. Gram staining

Following pure culture preparation, Gram staining was performed on all samples. The isolated bacteria were detected by light microscope. The bacteria was gram positive, rod shaped, single or in chains. Lactobacillus are gram positive which this gram staining results, represented that the isolated bacteria could be from Lactobacillus genus (Table 1).

3.1.2. Catalase test

During the catalase test in this study, no bubble was seen declaring that the isolated bacteria cannot intercede the decomposition of H_2O_2 to produce O_2 and they are catalase negative like Lactobacillus genus (Table 1).

3.1.3. Oxidase test

Lactobacillus are anaerobic or microaerophilic microorganism which indicated that they are oxidase negative. During this test no color changes was observed (Table 1).

3.1.4. TSI test

Each of the three sugars fermentation in the medium by Lactobacillus will produce acids, which will change the color of red (phenol red) to a yellow color. The result of TSI test revealed that all lactobacillus turned the medium to yellow (Table 1).

3.1.5. Motility test

The growth was restricted to the stab-line, and the surrounding medium was completely clear. This result indicated that they could be Lactobacillus genus as non-motile bacteria (Table 1) .

3.1.6. Carbohydrate fermentation test for Lactobacillus genus identification

For identification of Lactobacillus genus, carbohydrate fermentation test was performed and the result of 9 carbohydrate fermentation is shown in Table 2. The fundamental role of carbohydrate fermentation test is to explore the ability of bacteria to ferment a variety of carbohydrates. The indicator for this test is Phenol red broth base medium, based on bacteria scale of carbohydrate fermentation. Table 2. shows that all the isolates could ferment galactose, sucrose and lactose. None of them could ferment Rhamnose.

Table 1. Characteristics of the isolated strains.

Characteristics	I 1	I 2	I 3	I 4	I 5	I 6	I 7	I 8	I 9	I 10	I 11
Gram stain reaction	+	+	+	+	+	+	+	+	+	+	+
Catalase activity	-	-	-	-	-	-	-	-	-	-	-
Oxidase activity	-	-	-	-	-	-	-	-	-	-	
TSI test	+	+	+	+	+	+	+	+	+	+	+
Mobility test	-	-	-	-	-	-	-	-	-	-	-

I: Isolate

Table 2. Carbohydrate fermentation result for 11 isolates

Sample	I 1	I 2	I 3	I 4	I 5	I 6	I 7	I 8	I 9	I 10	I 11
Lactose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	+	+	+	-	+	+	+	+	+	+
Manitol	+	-	+	+	-	+	+	+	+	+	+
Sorbitol	-	+	+	+	-	+	-	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+

(-) Indicated no fermentation (Red), (+) Indicated fermentation and acid production (yellow)

3.1.7. Growth at different pH

According to Table 3, all isolates have different growth in different pH. The mean number of live Lactobacillus in samples with pH between 5-6 was significantly more than their numbers in the samples with pH of 2 to 4 and 9 to 10. The results of the Lactobacillus growth at different pH values indicated that optimum pH for growth of Lactobacillus is 5-6 to neutral environment (Table 3).

3.1.8. Bile salt tolerance

Results of bile salt tolerance tests are shown in Table 3. Probiotics should be able to show resistance to bile salts in the gastrointestinal tract. Most of Lactobacillus have this ability.

3.2. Molecular identification of Lactobacillus strains

PCR amplification of 16S rDNA was performed on DNA samples extracted from all bacterial strains. The nucleotide sequence was determined for all PCR amplicons, followed by Chromas sequence analysis and BLAST analysis at EZTaxon database which identified them all as members of Lactobacillus. rRNA gene sequence of isolates showed 99-100% identity to related bacterial sequences in the database (Table 4).

3.2.1. Result of sequence analysis of the 16S rRNA gene from isolated lactobacilli by Lactobacillus primer

The BLAST analysis was performed at EZTaxon and NCBI GenBank databases and the result for *rRNA* gene sequence of *L. paracasei* subsp.tolerans JCM 1171(T) was as below:

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTGATGATCGGTGCTTGC
ACCGAGATTCAACATAGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACA
GATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGT
ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC
GGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTG
GAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGA
GGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCC
GCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATCACCTGAGAGAT
CAGGTTTCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATGACTAGTTGC

Table 3. Lactobacillus growth at different pH and Bile salt agar tolerance

	I 1	I 2	I 3	I 4	I 5	I 6	I 7	I 8	I 9	I 10	I 11
Growth at pH 2 (c)	0	0	0	0	0	0	0	0	0	0	0
Growth at pH 3 (b)	0.189	0.177	0.181	0.182	0.183	0.181	0.175	0.177	0.179	0.180	0.182
Growth at pH 4 (b)	1.115	1.225	1.879	1.678	2.01	1.990	1.856	1.690	1.367	1.564	1.993
Growth at pH 5 (a)	3.688	3.670	2.950	3.245	3.585	3.545	3.575	3.685	3.425	3.125	3.570
Growth at pH 6 (a)	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
Growth at pH 9 (b)	0.189	1.996	1.634	1.212	1.456	1.554	1.358	1.709	1.342	1.793	1.808
Growth at pH 10 (b)	0.135	0.167	0.145	0.150	0.189	0.198	0.156	0.123	0.148	0.169	0.112
Growth at 0.3% oxgall	+	+	-	-	+	+	+	+	+	+	+

Values for growth at different pH are O.D. readings at 600nm. (a) shows very good growth , (b) shows very slightly growth, (c) shows no growth
(+) bile tolerance, (-) no bile tolerance

Table 4. Identities of 11bacterial isolates following the sequencing of 16S rRNA genes and BLAST analysis.

Isolate	Organism(s)with closest 16S rRNA gene sequence in GenBank	Accession no.	% Identity
1	*L. ozensis* strain Gon2-7	AB572592.1	100
2	*L. acidophilus* strain KU	AJ438156.1	100
3	*L. helveticus* strain D76	CP016827.1	100
4	*L. helveticus* strain Dpc 4571	CP000517	100
5	*L. fermentum* strain 1	FJ462686.1	100
6	*L. rossiae* strain DSM15814T	NZAKZK000	100
7	*L. casei* strain NCDO	D16550.1	100
8	*L. delbrueckii* strain ATCC 11842	CP002341	100
9	*L. crispatus* strain MRS 54.4	KX674035.1	99
10	*L. delbrueckii* strain SB3	KJ868758.1	100
11	*L. paracasei* subsp. tolerans JCM 1171(T)	D16550	100

3.2.2. Phylogenetic tree analysis

All principal nodes in the tree have high bootstrap support, showing accurate intergroup relationships. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences was constructed which shown the position of Strain H 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).

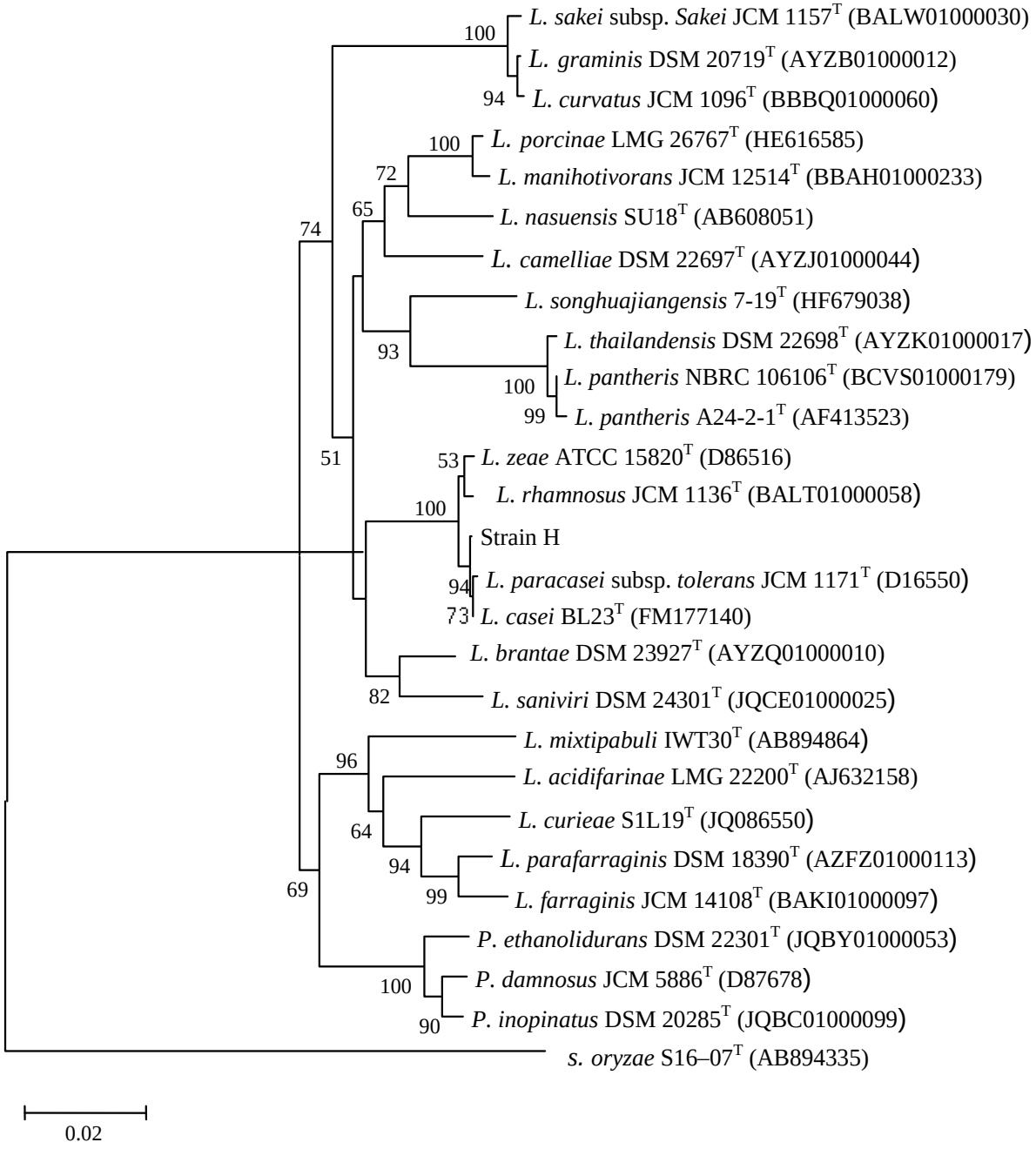

Figure 1. Phylogenetic tree according to 16S rRNA gene sequences

The sequence data of isolate (11): *L. paracasei* subsp. tolerans JCM 1171(T) to GenBank. GenBank accession number(s) for this nucleotide sequence(s) is submitted: SUB3536942 Strain MG818769.

3.3. B-group vitamin measurement by HPLC analysis

Results of HPLC analysis of the supernatant samples generated following bacterial culture centrifugation are given in Table 5. *L. fermentum* strain 1 with 521.7 µg mL^{-1} of vitamin B_2 produced the highest amount of vitamin B_2 (Figure 2). All of the isolates produced vitamins B_3, B_6 and B_9 to different levels. The highest level for vitamin B_3 comparing to standard (Figure 3) produced by *L. acidophilus* with 522.7 µ mL^{-1} (Figure 4). The calibration curve for vitamin B_3 is shown in (Figure 5).

The highest levels of vitamin B_6 and B_9 was produced by *L. paracasei* subsp. tolerans JCM 1171 (T). The highest level of vitamin B_6 comparing with vitamin B_6 standard (Figure 6).

Is 1566.17 µg ml^{-1} (Figure 7) and the calibration curve for vitamin B_6 is shown in (Figure 8). Chromatograms of vitamin B_9 standard displayed in (Figure 9) and produced vitamin B_9 by *L. paracasei* in (Figure 10) with 1279.72 µg mL^{-1} and calibration curve of vitamin B_9 in (Figure 11). The below chromatograms shows the highest production of vitamin B_2, B_3, B_6 and B_9 compared to vitamins standard.

3.3.1. Chromatogram of produced vitamin B_2 by *L. fermentum*

The result of HPLC of supernatant was *L. fermentum* which could produce vitamin B_2 in culture medium. The colorful peak refers to standard vitamin B_2 and the black peak refers to sample number 5 (Figure 2). The result showed the peak of vitamin B_2 is similar to standard vitamin B_2.

3.3.2. HPLC Chromatogram of standard vitamin B_3 and produced vitamin B_3 by *L. acidophilus* and calibration curve

Figure 3 showed the HPLC Chromatogram of Standard vitamin B_3 with Ret. Time: 3.555 min and Area: 7701088 mAu/min which had injected to HPLC as external standard with the concentration of 270 µg mL^{-1}. Figure 4 showed the chromatogram of the highest production vitamin B_3 produced by *L. acidophilus*. By comparing the two chromatograms which showed the similar characteristics, the concentration of vitamin B_3 was calculated. Calibration curve of vitamin B_3 was drawn by Excel program. Y= aX±b is equation of straight line which Y is UV absorption and X is concentration of standard vitamin B_3 (Figure 5).

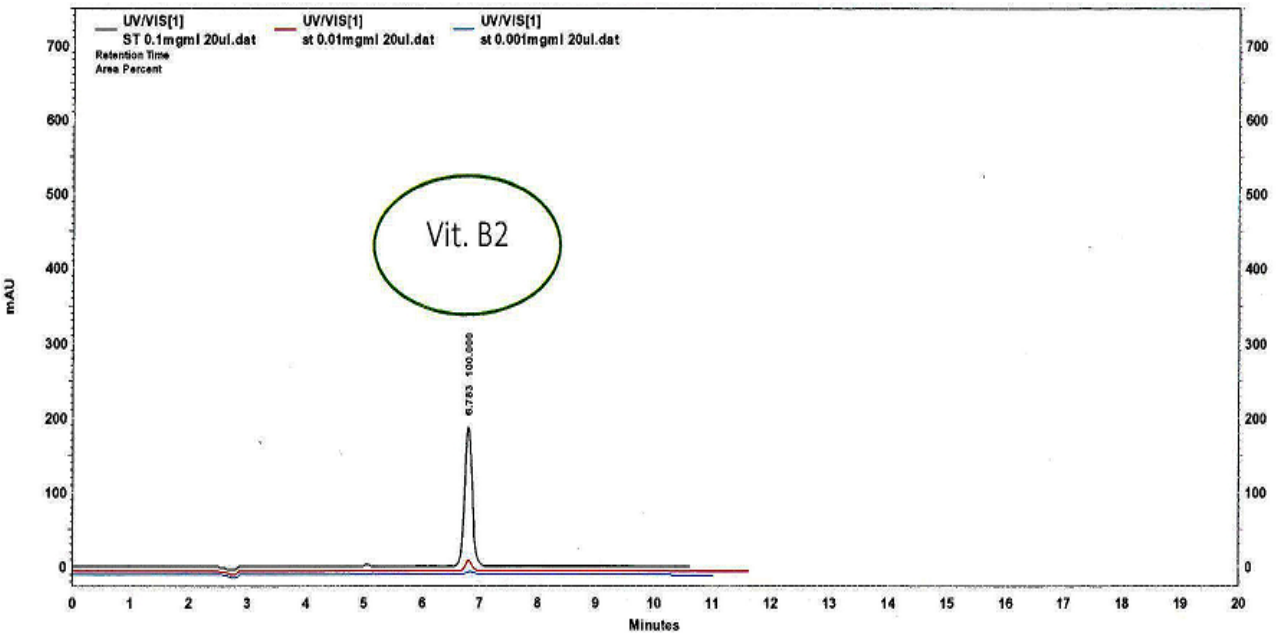

Figure 2. Chromatogram of the highest production of vitamin B_2 by *L. fermentum*. Colorful peaks refer to standard samples and black peak refers to sample number 5 (*L. fermentum)*.

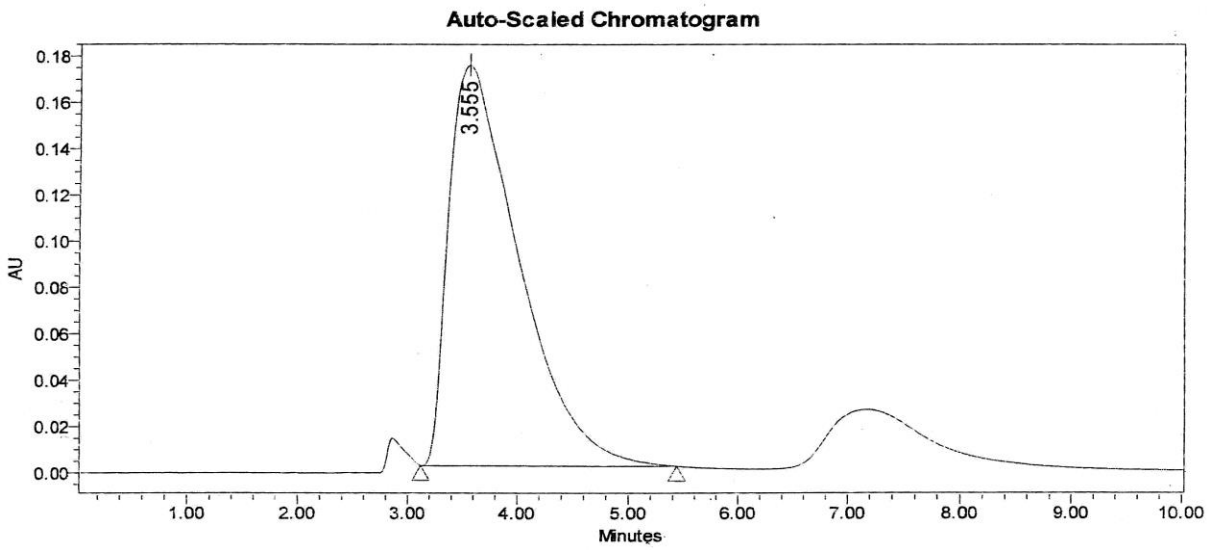

Figure 3. Chromatogram of vitamin B_3 standard, Ret. Time: 3.555 min, Area: 7701088 mAu min^{-1}

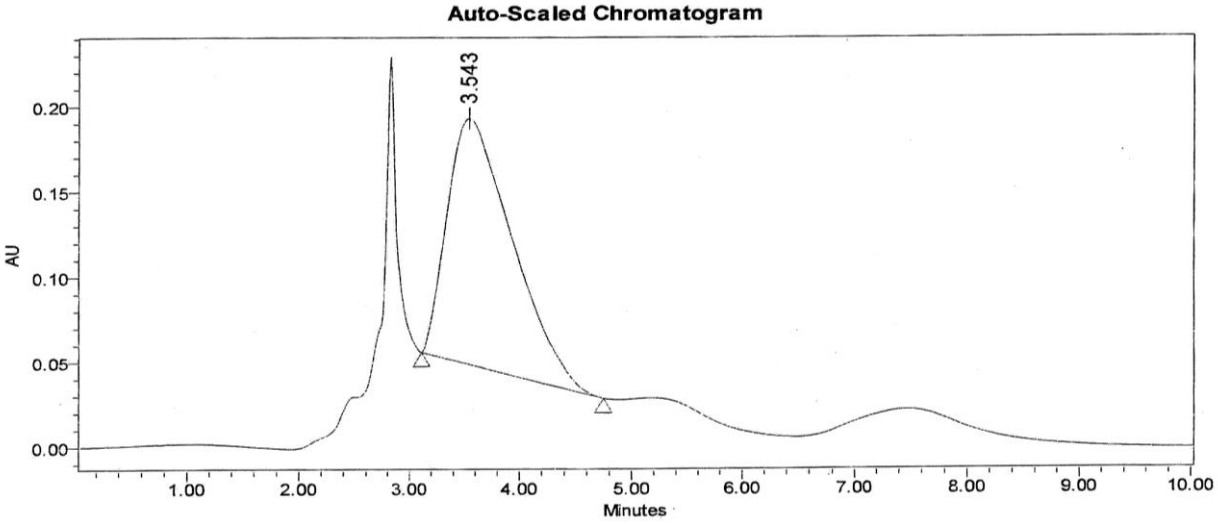

Figure 4. Chromatogram of the highest production of vitamin B_3 by *L. acidophilus*. Ret. Time: 3.543 min, Area: 5963210 mAU min^{-1}

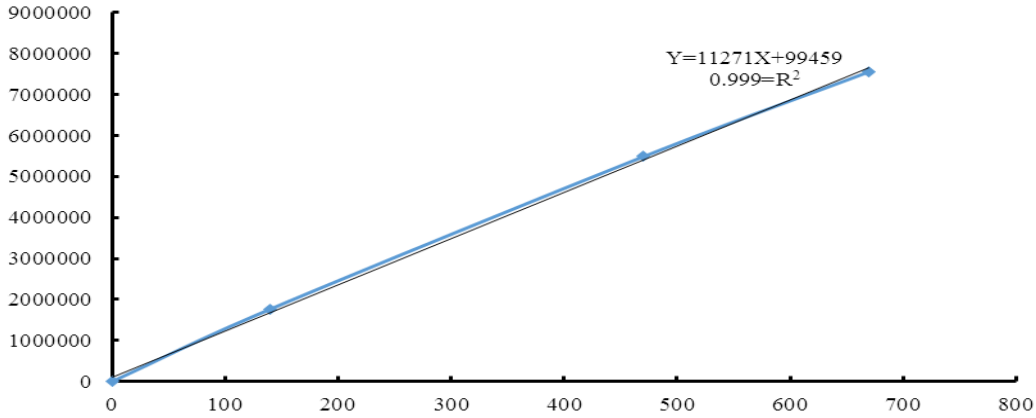

Figure 5. Calibration curve of vitamin B_3, X= Concentration of vitamin B_3 standard, Y= UV absorption, R^2= coefficient of determination

3.3.3. HPLC Chromatogram of vitamin B_6 standard and produced vitamin B_6 by *L. paracasei* and calibration curve

Figure 6 showed the chromatogram of vitamin B_6 standard with Ret. Time: 5.451 min and Area: 6174510 mAU min^{-1} which had injected to HPLC as external standard with the concentration of 298 µg mL^{-1}.

Figure 7 showed the chromatogram of the highest production vitamin B_6 produced by *L. paracasei*. By comparing the two chromatograms the concentration of vitamin B_6 was calculated. Calibration curve of vitamin B_6 was drawn by Excel program. Y= aX±b is equation of straight line which Y is UV absorption and X is concentration of vitamin B_6 standard (Figure 8).

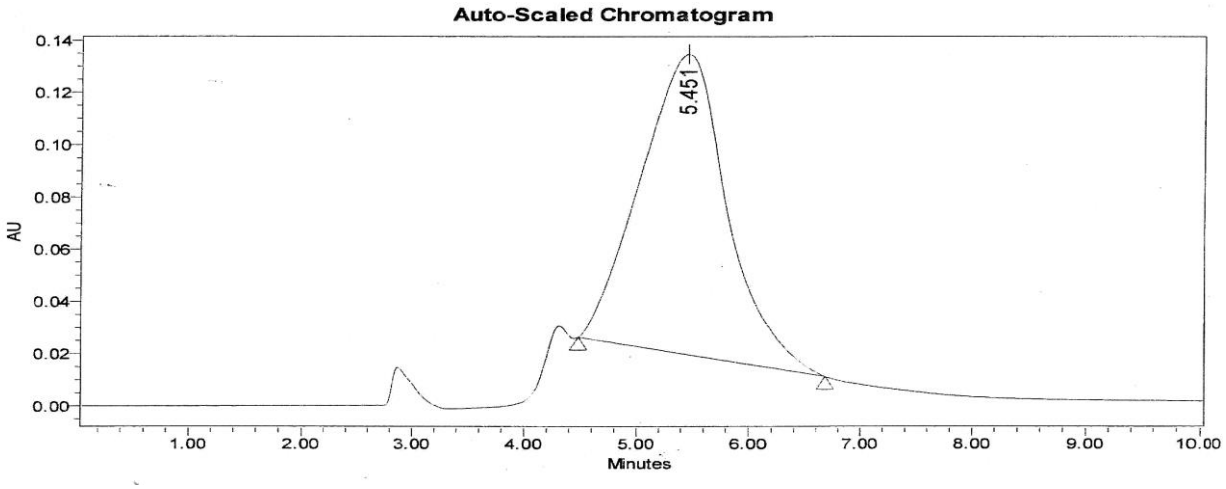

Figure 6. Chromatogram of vitamin B_6 standard, Ret. Time: 5.451 min, Area: 6174510 mAU min^{-1}

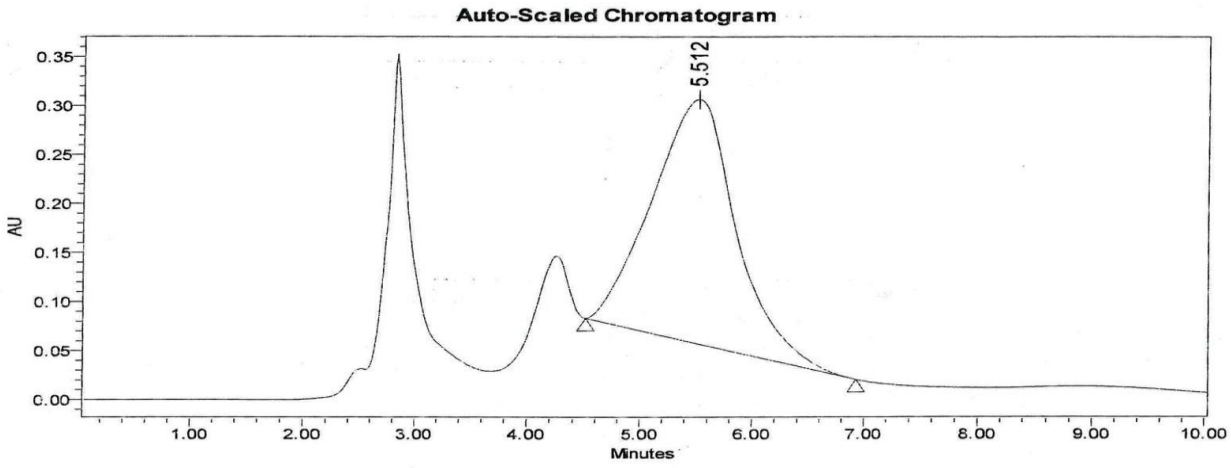

Figure 7. Chromatogram of the highest production of vitamin B_6 by *L. paracasei*, Ret. Time: 5.512 min, Area: 12980349 mAU min^{-1}

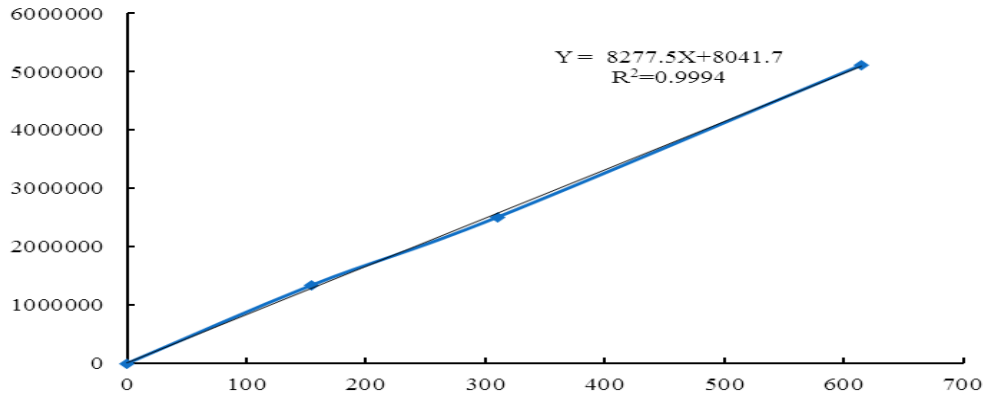

Figure 8. Calibration curve of vitamin B_6, X= Concentration of vitamin B_6 standard, Y= UV absorption, R^2= coefficient of determination

3.3.4. HPLC Chromatogram of vitamin B_9 standard and produced vitamin B_9 by *L. paracasei* and calibration curve

Figure 9 showed the chromatogram of vitamin B_9 standard with Ret. Time: 4.973 min, Area: 685859 mAU min^{-1} which had injected to HPLC as external standard with the concentration of 42 µg ml^{-1}. Figure 10 showed the chromatogram of the highest production vitamin B_9 produced by *L. paracasei*. By comparing the two chromatograms the concentration of vitamin B_9 was calculated. Calibration curve of vitamin B_9 was drawn by Excel program. Y= aX±b is equation of straight line which Y is UV absorption and X is concentration of vitamin B_9 standard (Figure 11).

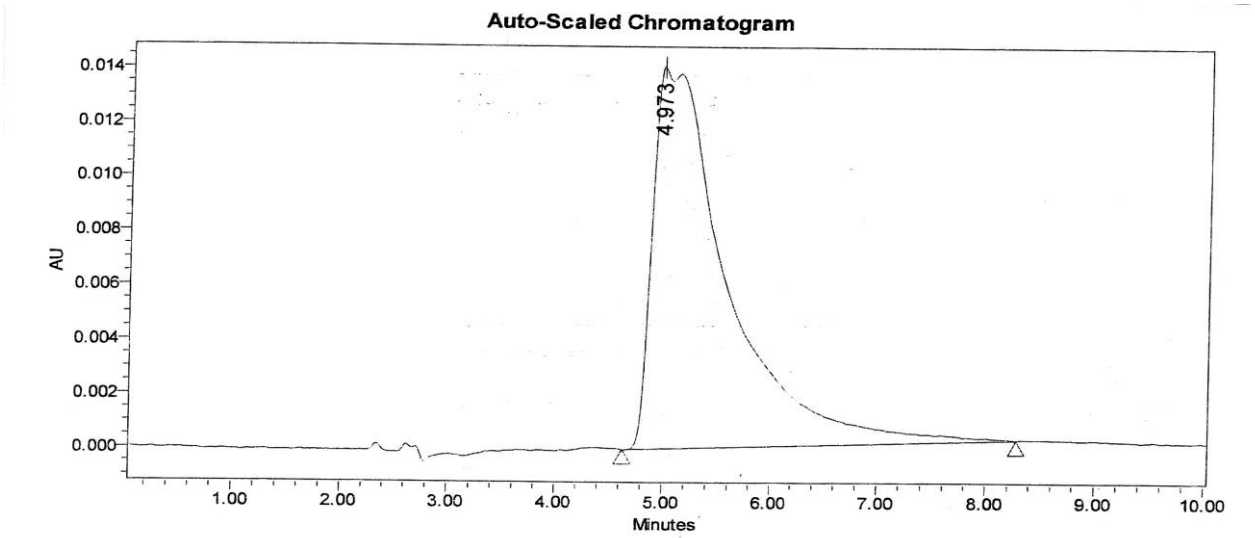

Figure 9. Chromatogram of vitamin B_9 standard, Ret. Time: 4.973 min, Area: 685859 mAU min^{-1}

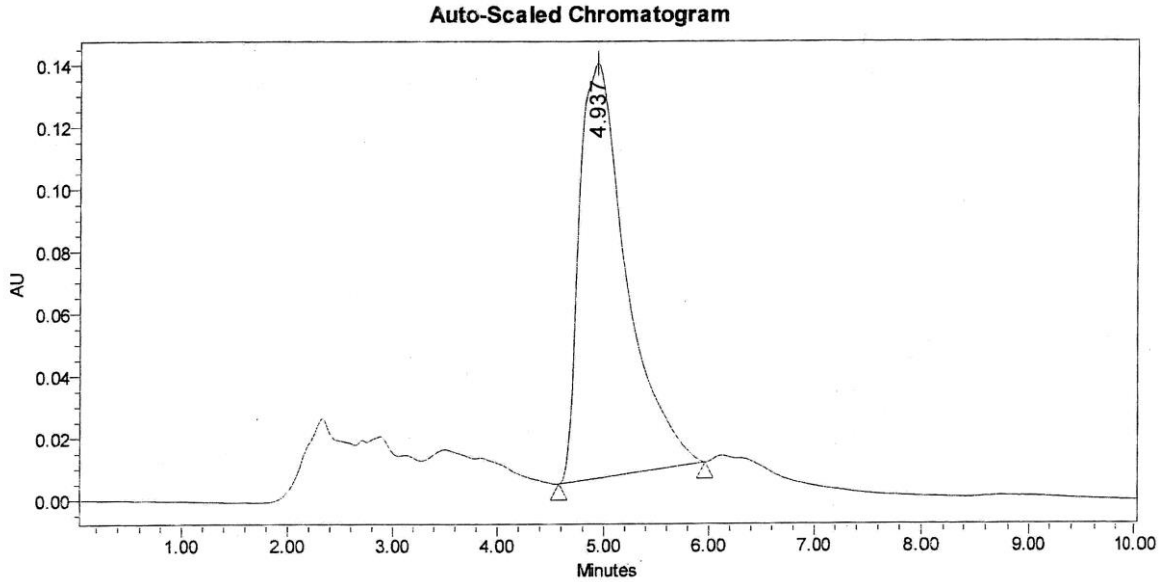

Figure 10. Chromatogram of the highest production of vitamin B_9 by *L. paracasei***,** Ret. Time: 4.937 min, Area: 4179560 mAU min^{-1}

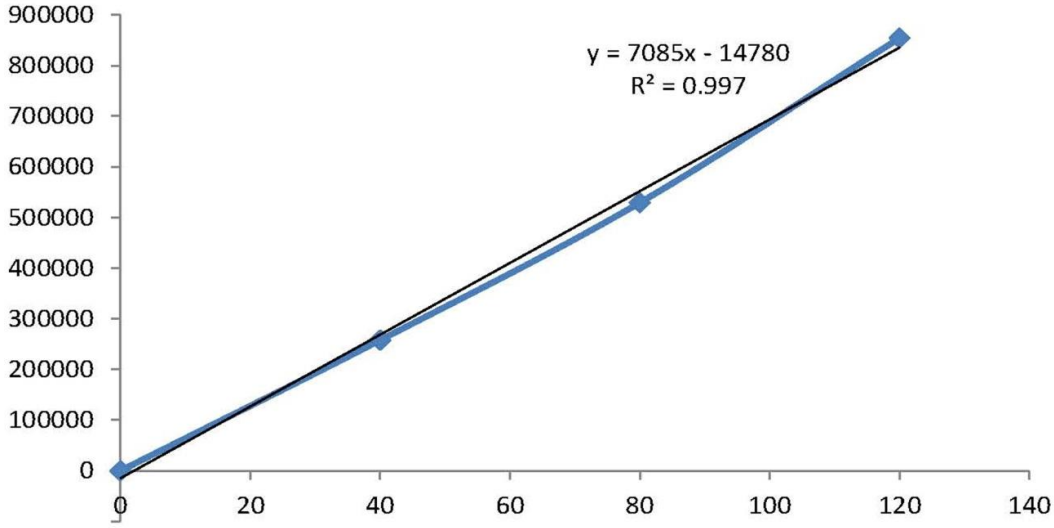

Figure 11. Calibration curve of vitamin B_9, X= Concentration of vitamin B_9 standard, Y= UV absorption, R^2= coefficient of determination

Concentration of each vitamin B produced from each lactobacillus calculated with this formula:
Concentration of B_3 standard: 270 µg ml^{-1}
Concentration of B_6 standard:298 µg ml^{-1}
Concentration of B_9 standard: 42 µg ml^{-1}

$$C_{VitB} = \frac{\text{Area of sample}}{\text{Area of std}} \times \frac{\text{Concertration of std}}{\text{Volume of sample}} \times \text{total volume} = \mu g\ ml^{-1}$$

Table 5. The result of amount of each vitamin (B_3, B_6 and B_9) produced by each isolates

Vitamin	I 1	I 2	I 3	I 4	I 5	I 6	I 7	I 8	I 9	I 10	I 11
B6	5.69	60.58	6.19	8.88	67.46	20.44	21.62	24.88	21.12	22.34	1566.17*
B3	2.57	522.7*	3.50	4.81	521.7	8.92	15.03	2.82	15.39	17.37	0.57
B9	3.70	30.81	3.44	2.74	26.84	3.53	2.96	2.87	2.77	2.74	1279.72*

Units are in µg mL^{-1}.
*Show the significant different

Table 6. ANOVA for produced vitamin B_3, B_6, and B_9 from samples 1 to 11

	df	Mean Square	F	Sig.
B6	10	8643205.159	7.253E35	0.000
B3	10	173273.192	3.406E36	0.000
B9	10	5883042.040	4.499E33	0.000

According to Table 5, all samples (1-11) produced different levels of vitamin B_3, B_6 and B_9. Statistical analysis for levels of produced vitamin B group showed (Table 6) that all samples (1-11) produced different levels of vitamin B_6 so that *L. paracasei* subsp. tolerans JCM 1171(T) showed the highest production of vitamin B_6 ($p \leq 0.01$). All samples produced different levels of vitamin B_3 and the highest amount significantly produced by *L. acidophilus* strain KU ($p \leq 0.01$). With regard to vitamin B_9, *L. paracasei* subsp. tolerans JCM 1171(T) showed the highest production ($p \leq 0.01$).

Lactic acid bacteria represent a gram positive, non-sporulating, anaerobic or microaerophilic rod shape or coccobacilli. Lactic acid is one of the major fermentation

products of carbohydrates metabolism with increasing important and health-related properties by LAB. [2,5]. Lactobacillus is the widest and major genus of lactic acid bacteria, comprising species with several physiological and biochemical characterization and resistance to acidic environment. Members of Lactobacillus genus are being applied in main industrial productions [3].

B-group vitamins play important roles in human health and survival. Biosynthetic capacity of most vitamins for human is low and must be provided from external resources [9]. However due to malnutrition, vitamin production by LAB has recently gained the attention of the scientific community. Most strains of LAB can produce B-group vitamins. Most of LAB isolated from different type of dairy products have been demonstrated to produce a variety amounts of B-group vitamins [11,26,27]. Using special transporters in the cell membrane or through cellular lysis, these vitamins are usually stored inside the cells and extracted by straight diffusion [28]. Among LAB members, the genus Lactobacillus seems to possess a greater potential for the B-group vitamins production, as they have been shown to be health-promoting for humans. In addition to several members, with vitamin-producing capability, having been isolated from different dairy products [29,30,11], recent genetic investigations have provided clues on Lactobacillus genes being involved in vitamin biosynthesis. Some of these include the direct association of higher riboflavin concentrations with significant increases in the expression of ribA, ribB, and ribC genes in *L. plantarum* [31]. The recently completed genome sequence of *L. fermentum* has revealed the presence of genes needed for the synthesis of vitamins similar to B_1, B_2, B_5, B_7 and B_9 [32]. Other similar studies include those of Li et al. and Suryavanshi et al. in *L. plantarum* [33,34].

In the present study, it was aimed to identify the Lactobacillus strains capable of producing B-group vitamins namely B_3, B_6 and B_9 from traditional yogurt samples consumed in different regions of Iran. HPLC is a method commonly applied to separate, detect, and quantify each component in a mixture combination such as food and drug samples. Following isolation and identification of several Lactobacillus strains in Iranian yogurt samples, HPLC analysis revealed the significantly different levels of vitamin B_3, B_6 and B_9 produced by these bacteria.

4. Conclusion

Nowadays, humans are becoming more aware about their health and economically wise, not to mention the focusing on environmental factors, the use of vitamin producer LAB could suggest a simple benefit over chemical synthesis by expanding the nutritional value of food, while being an economical alternative to present vitamin enrichment programs. In addition, designing vitamin-fortified functional foods by using vitamin-supplying lactic acid bacteria is a feasible approach. We believe that our findings along with those of the research community will pave the way for reaching this goal.

5. Acknowledgements

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6. Conflict of Interest

None of the authors had any personal or financial conflict of interest.

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تولید ویتامین‌های $B_{۳}$، $B_{۶}$ و $B_{۹}$ توسط لاکتوباسیلوس جدا شده از نمونه‌های ماست سنتی ۳ شهر ایران در طول زمستان

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چکیده

سابقه و هدف: ویتامین‌های گروه ب در بسیاری از متابولیسم‌های درون سلول نقش مهمی دارند و انسان نمی‌تواند این ویتامین‌ها را تولید کند. از این رو، باید از منابع خارجی دریافت شوند. هدف این پروژه فراهم کردن فهم جدیدی برای ارزیابی تولید ویتامین‌های $B_{۳}$، $B_{۶}$ و $B_{۹}$ توسط لاکتوباسیلوس جدا شده از نمونه‌های ماست سنتی ۳ شهر ایران شامل گلپایگان، سنندج و تهران (دماوند) می‌باشد.

مواد و روش‌ها: پس از کشت بی‌هوازی ۷۲ ساعته باکتری‌های اسید لاکتیک در ۳۷°C در ۵ درصد دی اکسید کربن، بعضی از گونه‌های لاکتوباسیلوس‌ها از ماست‌های سنتی جدا و با روش‌های ریخت‌شناسی و بیوشیمیایی شناسایی شدند. گونه‌های لاکتوباسیلوس جدا شده با روش‌های مولکولی 16S rRNA، PCR و توالی‌یابی نیز شناسایی شدند. این گونه‌ها شامل: *Lactobacillus ozensis* strain Gon 2-7، *Lactobacillus acidophilus* strain KU، *Lactobacillus helveticus* strain D76، *Lactobacillus helveticus* strain Dpc4571، *Lactobacillus casei* strain NCDO، *Lactobacillus rossiae* strain DSM15814T، *fermentum* strain1 *Lactobacillus* strain SB3، *Lactobacillus crispatus* strain MRS 54.4، *Lactobacillus delbrueckii* strain ATCC 11842 و *Lactobacillus delbruckii* *Lactobacillus paracasei* sub. tolerans JCM1171(T) بودند. توالی paracasei sub. tolerans JCM1171(T) در سایت NCBI ثبت شد. توانایی تولید ویتامین‌های گروه ب توسط این باکتری‌ها با روش کروماتوگرافی مایع با کارایی بالا بررسی شد. میزان تولید ویتامین‌های $B_{۳}$، $B_{۶}$ و $B_{۹}$ با آزمون تحلیل واریانس (ANOVA) بررسی شد.

یافته‌ها و نتیجه‌گیری: ۱۱ گونه لاکتوباسیلوس جدا شده از نمونه های ما ست سنتی شناسایی شدند. شرایط بهینه برای رشد لاکتوباسیلوس‌ها، pH بین ۶-۵ تا خنثی (5-6 to neutral environment) و دمای ۴۰-۳۷°C بود. تمامی ۱۱ گونه لاکتوباسیلوس جدا شده از ماست‌های سنتی ویتامین‌های $B_{۳}$، $B_{۶}$ و $B_{۹}$ را در مقادیر مختلف تولید می‌کردند. *Lactobacillus paracasei* sub. tolerans JCM1171 (T) بیشترین مقدار تولید ویتامین‌های گروه ب را داشت. تولید ویتامین $B_{۶}$ (۱۵۶۶/۱۷ $\mu g\ ml^{-1}$) و ویتامین $B_{۹}$ (۱۲۷۹/۷۲ $\mu g\ ml^{-1}$) توسط این گونه معنی‌دار بود ($P\leq ۰/۰۱$) و *Lactobacillus acidophilus* strain Ku بیشترین میزان تولید ویتامین $B_{۳}$ (۵۲۲/۷$\mu g\ ml^{-1}$) را داشت. بر اساس یافته‌های به‌دست آمده، تمام ۱۱ گونه لاکتوباسیلوس جدا شده در این مطالعه توانایی تولید برخی از ویتامین‌های گروه ب را دارند مانند *Lactobacillus fermentum* که بیشترین میزان ویتامین $B_{۲}$ را تولید کرد. این لاکتوباسیلوس‌ها می‌توانند ویتامین‌های گروه ب را به صورت طبیعی و اقتصادی به جای تولید شیمیایی و صنعتی تولید کنند. این گونه‌ها می‌توانند جایگزینی طبیعی و مقرون به صرفه برای برنامه‌های پرهزینه غنی‌سازی ویتامین‌ها باشند. گونه‌های لاکتوباسیلوس شناسایی شده در این تحقیق، به‌خصوص *Lactobacillus paracasei* می‌تواند برای بهبود فرآورده‌های جدید تخمیری غنی شده با ویتامین گروه ب و جایگزینی غنی‌سازی و مکمل‌یاری با ویتامین‌های سنتزی بحث برانگیز باشند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- ویتامین‌های گروه ب
- کروماتوگرافی مایع با کارآیی بالا
- لاکتوباسیلوس
- پروبیوتیک
- ماست

***نویسنده مسئول**

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