Biomacromol. J., Vol. 7, No. 1, 55-59, July 2021.

Biomacromolecular Journal
www.bmmj.org

Evaluation of Vitamin B_2 Production by Lactobacilli Isolated from Local Iranian Cheeses

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(Received 1 September 2019, Accepted 24 December 2021)

ABSTRACT

In general, vitamin B plays an important role in the cell's metabolic cycle. It stimulates and promotes cell growth and division. Lactobacillus is valued today for its important role in gastrointestinal health and its ability to produce vitamins, including the vitamins B group. These probiotic bacteria are used as initiators in dairy products, especially traditional cheeses. In this study, 16 cheese samples were collected from Markazi province in Iran and their lactobacilli bacteria were isolated. Molecular identification of Lactobacillus species was performed by the 16S rRNA method using specific primers. The identified bacteria (five species of Lactobacillus) were examined for production of vitamin B_2. All isolates were able to produce vitamin B_2, the highest level by the L. fermentum strain SK152 was 285.8 µg ml^{-1}, while the lowest level was 21.42 µg ml^{-1} produced by L. delbruckeii subsp strain: YIT 0080. According to our results based on the differences in vitamin B_2 production on isolated bacteria from local cheeses and the important role of these micronutrients in human life, it is recommended to consume traditional cheeses as a good source of vitamin B groups in daily regimen.

Keywords: Vitamin B_2, HPLC, Lactobacillus, Native cheeses

INTRODUCTION

Traditionally made from raw milk, cheese has a wider and more pronounced range of nutrients [1]. The properties of these cheeses are clearly due to the local species, diversity, and the unique microbial flora of the milk used to make this type of cheeses [2]. Lactobacillus has been identified and introduced as the most common organism used in the manufacture of probiotic products. Lactobacillus is the major species of cheese made from raw milk [3] and [4] the B vitamin groups are generally involved in the cell's metabolic cycle, thus boosting the body's metabolism and promoting cell growth and division. These vitamins are involved in the proper functioning of the immune and nervous systems and are essential for healthy skin and muscles. B vitamins reduce stress, depression, and skin and nerve health [5]. Vitamin B_2 enters the body with food and is transported to the bloodstream via transporters in the intestinal wall. The amount of this vitamin needed by the human body is 1.5 mg daily. Vitamin B_2 is also involved in the activation of vitamins B_3 and B_6. Riboflavin (vitamin B_2) releases energy from carbohydrates, proteins, and fats. It is also important for the regeneration, growth and repair of skin, hair, nails and joints. This vitamin destroys UV rays, heals wounds on the mouth, lips and tongue, and enhances the functioning of the immune system [6]. Markazi province, which is located in the middle of Iran, is experiencing the emergence of a wide variety of traditional foods, especially dairy products, due to the diversity of the environment and the presence of different ethnic groups. In this research, the traditionally prepared local cheeses were evaluated for presence of Lactobacilli and their ability in producing of vitamin B_2. The purpose of this study was to identify Lactobacillus species isolated from traditional cheese to develop pure primers for vitamin B_2 production.

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MATERIALS AND METHODS

Chemicals and Reagents

The culture media such as MRS broth and MRS agar

were purchased from local markets with Difco & BBL Laboretories brand. HPLC-grade methanol, Vitamin B_2 reference standard and other chemical were prepared from Sigma-Aldrich. The HPLC and its applications will be explained in the related section.

Sample Preparation

In this study, 16 traditional cheese samples from Markazi Province in Iran, including Ashtian, Save, Tafresh, and Farahan cities, were prepared and as 150 grams were stored at sterile conditions. First, 5 g of cheese was added to 45 ml of sterile 2% sodium citrate solution at 45 °C, and then homogenized for 1 min. Then, after 24 h of anaerobic culture, lactic acid bacteria were linearly cultured on MRS broth and MRS agar media, and placed at 5% carbon dioxide incubator at 37 °C. Isolated bacteria were examined for morphological and biochemical properties by standard bacteriological methods including thermal staining, oxidase, catalase, motion, and sugar fermentation tests. Bacteria have also been tested for several probiotic properties, including resistance to acids and bile.

Detection of Lactobacilli by Polymerase Chain Reaction (PCR)

Pure culture and the isolated Lactobacilli were recognized by PCR techniques. The following steps were performed to assess the genus of Lactobacillus obtained in the 16S rRNA sequence, respectively [7,8].

Genome extraction processes. Each isolated bacterium was first cultured in Luria broth (LB) medium and maintained at 37 °C for 18 h. DNA extraction begins when the turbidity of the tube is 2 McFarland or 6×10^8 CFU. The sample was then transferred to a 2 ml Eppendorf tube and centrifuged at 25 °C for 2 min at 13000 rpm. The supernatant was then discarded to obtain sufficient precipitate from the bacteria. 20 microliters of lysozyme were added to this bacterial precipitate and placed in a shaker at 100 rpm for 5 h [9].

PCR reaction. The extracted DNA sample was taken out of the freezer and thawed at room temperature. The PCR microtubes were then numbered in sample order. For more accurate identification, we examined negative controls that included all the materials needed for the PCR reaction except the sample DNA. The purpose of using the negative control is to check for contamination of the PCR solution with external DNA. If the PCR solution is not contaminated with foreign DNA, no bands should form on the agarose gel of the negative control sample after PCR. The PCR reaction was performed in the final volume of 25 µl. PCR reaction using specific primers (universal primer 27F, 1492R) of the 16S rRNA gene was amplified in the Thermocycler Ferrotec, Japan [11,10]

DNA extraction from agarose gel. To extract DNA from agarose gels, we used a DNA extraction kit called the Biospin Gel Extraction Kit. The kit included elution buffer, wash buffer, extrusion buffer, and spin column. First, the DNA-containing fragment was cut from an agarose gel with a sharp sterile razor blade and transferred to a 1.5 ml microtube. After receiving the results, the sequence was extracted with Chromas software and checked it in the EzTaxon database. The PCR results were sequenced at the NCBI site.

Analysis of Vitamin B2 by HPLC-UV

Preparation of samples for analysis. The identified strains were cultured in MRS broth for 72 h, one ml of culture medium was removed and combined with one ml molybdenum phosphate buffer containing 0.5% sodium ascorbate. The capacity was increased as a mobile phase, and it was heated in a hot water bath at 90 °C for 15 min. The sample was then centrifuged for 10 min to separate the supernatant. The supernatant was then passed through a 0.22-micron syringe filter to remove Lactobacillus. To confirm that the ruby solution was sterile, 100 µl was recultured in MRS broth medium and no growth was observed after 72 h. After preparing the sterile supernatant, the sample was injected into HPLC.

HPLC conditions. The applied instruments for analysis was HPLC Waters e2695 coupled with UV detector. The HPLC column was silica Waters Symmetry reverse-phase C18, 250 mm lenth, 4.6 mm diameter, 5 µm spherical particle sizes, the column oven set at 25 °C. The mobile phase was methanol: deionized water (22:78) with flow rate 1 ml min^{-1}. The injectin volumes of samples were 20 µl after using 0.22-micron syringe filter. The used wavelength of detector was 290 nm for Vitamin B_2.

Determination of vitamin B_2. The calibration curve of vitamin B_2 were drawn with the results of area under curve

(AUC) versus different concentration of vitamin B_2 150, 300, 600, 800 µg ml^{-1}. The slope, Coefficient of determination and line equation were determined and the concentration of vitamin B_2 for each sample wich extracted from the broth mdium of bacteria were calculated based on the calibration curve data. In this study we used ISO ISIRI number 5333 for the calculation and also Empower software for HPLC analysis.

RESULTS AND DISCUSSION

PCR Results

PCR results, sequencing at NCBI sites, and use of BLAST as a basic tool for finding local overlaps, followed by extraction and analysis of Nucleotide Blasts using Chromas software in the EzTaxon database. Table 1 shows the identified Lacticaseibacillus genus and strains. Lactobacillus bands using 16S rRNA primers were observed by PCR and gel formation, and bands were formed from five isolated samples of Lactobacillus obtained by pure culture.

Table 1. Lactobacillus Strains Isolated from Traditional Cheeses of Markazi Province

Strain	Microorganism with the closest sequence 16S rRNA	Accession no	Similarity
1	Lactobacillus delbrueckii subsp strain: YIT 0080	AB008207.1	99
2	Lactobacillus fermentum strain SK152	CP016803.1	100
3	Lactobacillus plantarum strain MSD1-4	MH620395.1	100
4	Lactobacillus acidophilus strain LAG8011	CP054559.1	98
5	Lacticaseibacillus casei strain J026	GQ395613.1	100

Lactobacillus fermentum strain SK152, a sequence of strain 2, was recorded at gene bank. The nucleotide accession number for this bacterium is CP016803.1. After extraction, the concentration of the extracted DNA was measured by spectrophotometric method and the quality of the extracted DNA was evaluated by agarose gel electrophoresis.

Evaluation of Vitamin B_2 Production Capacity by Lactobacilli Strains

Standard control sample was used to evaluate the production of vitamin B_2 by individual isolated strain. The results of the HPLC analysis were chromatograms which conformed to vitamin B retention time in comparison with standards. Chromatograms peak retention times were measured relative to the standard in which all samples have been injected. All lactic acid bacteria isolated from Markazi cheese samples were produced vitamin B_2. Figures 1 and 2 show chromatograms of vitamin B_2 standard and produced by an isolated Lactobacillus. Using the resulting linear equation, the height or area value below the unknown sample peak is calculated as the exact value for the analyte [12,13].

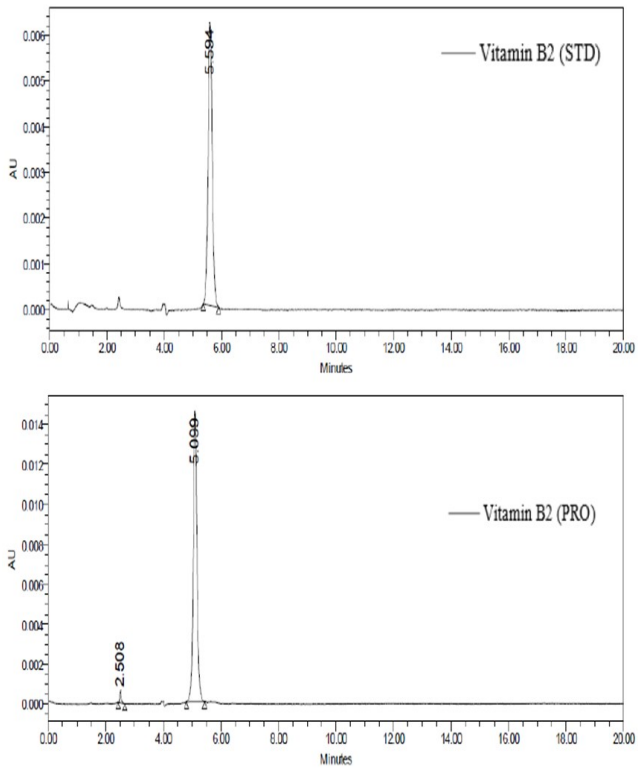

Fig. 1. Chromatograms of Vitamin B_2 standard and produced by L. Fermentum

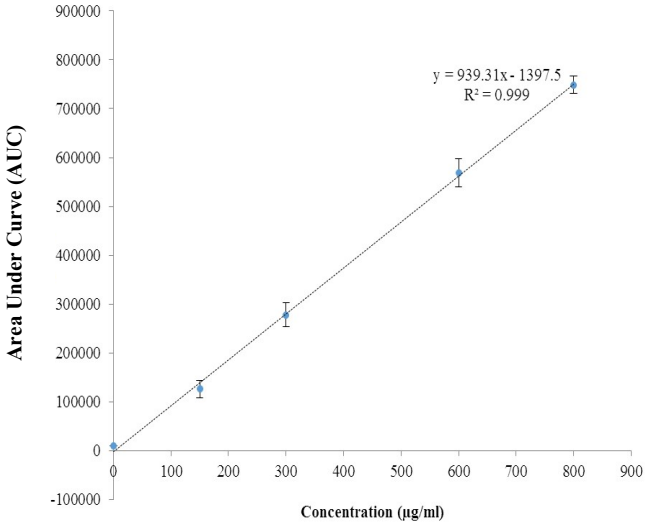

Fig. 2. Calibration curve of standard solutions of vitamin B_2 (n = 3).

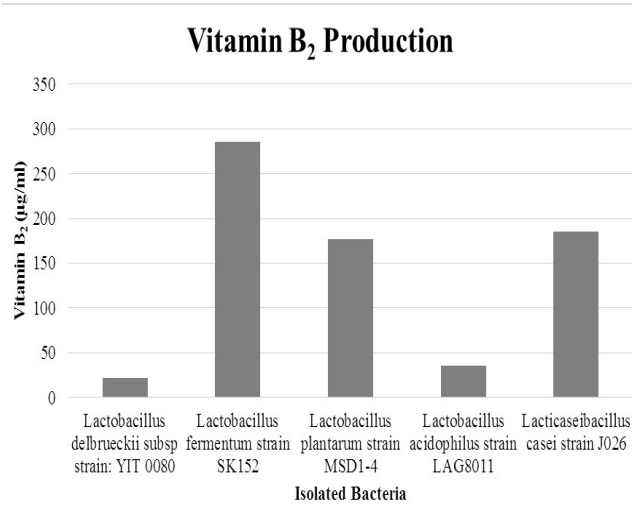

Fig. 3. Amounts of produced vitamin B_2 by Lactobacillus strains (µg ml^{-1}).

Chromatograms of vitamin B_2 standard and produced by L. Fermentum. The results of HPLC analysis is shown in Fig. 1. Chromatograms of vitamin B_2 standard and produced by L. fermentun were in sharp peak and desired shape. The other chromatograms also were in well-shaped. The calibration curve of standard solutions of vitamin B_2 is demonstrated in Fig. 2. Linearity between the aquired data was in an acceptable criterion.

Results of production of vitamin B_2 by isolated Lactobacilli. Figure 3 shows the comparison of vitamin B_2 production between the isolated Lactobacilli. The highest production was resulted by Lactobacillus fermentum strain SK152 as amount of 285.8 µg ml^{-1}, while the lowest belong to Lactobacillus delbrueckii subsp strain YIT 0080 as 21.42 µg ml^{-1}.

The purpose of this study is to investigate the production of vitamin B_2 by bacteria isolated from traditional cheese, enabling the study and acquisition of bacteria with appropriate industrial technology in various fields. Vitamin B_2 are essential and needed by the body and humans, so they cannot be synthesized and must be obtained from external sources that can provide these vitamins easily and inexpensively. It is important that the body prepares for improving health and the economy [14].

In this study, different species of Lactobacillus were isolated from multiple samples of different cheese samples in the Markazi province, similar to the study by Mildamadi *et al*. They isolated strains of lactic acid bacteria from traditional Iranian dairy products for bacteriocin production against eight standard strains of Gram-positive, Gram-negative, and yeast. Eight native Lactobacillus strains were selected for production, two of which were considered for further study. Their method for assessing vitamin B_2 from Lactobacillus lactobacillus was similar to present study. [15] Leblanc J.G. *et al.* in 2011 published a paper on the production of B vitamins by lactic acid bacteria and their effectiveness. They have shown that it makes economic sense to use vitamin-forming lactic acid bacteria in foods as an initiator, which is consistent with our results. They also recognized that B vitamins are the most common and important vitamins produced by lactic acid bacteria [16]. Another study, Lactobacillus plantarum, isolated bacteria from milk and bread as an in vitro production strategy for the vitamin Mattia pia. To investigate the probiotic properties of L. plantarum CS23 as in this study, they found that the bacteria were exposed to pH 2.5 and bile salts for up to 3 h and a high proportion of them survived. We used 0.3 Ox Gall and pH 2.1. They found that bacteria could attach to the enterocytes of 17 CFU/Caco2, which had the riboflavin peroneum on it, which caused the biosynthesis of this vitamin [17]. In a similar study by Tamaskini *et al*.,

they compare folic acid production using probiotic yogurt and regular yogurt, and the natural bacterium Lacticaseibacillus casei can increase folic acid production by up to three times the amount produced by regular yogurt. They also used high-performance liquid chromatography to measure vitamin B_9 levels. The methods and results for isolating vitamin B_2 were consistent with the methods and results of us [18]. In 2016, Ping Li *et al* examined the ability of bacteria to produce vitamins B_9 and B_2 in a manner similar to us with HPLC, and the ability of vitamin B_2 production, the genetic structure of bacteria with gene clusters for synthesis. A vitamin, the probiotic properties of this bacterium are due to this gene group. [19] In 2011, Sathyaarayanan J. *et al.* used the MRS medium to optimize riboflavin production by Lactobacillus fermentum isolated from yogurt. They examined MRS components such as peptone, meat extract, glucose, and K_2HPO_4 as variable parameters. They found that an increase in meat extract, glucose and K_2HPO_4 had a significant positive effect on the production of vitamin B_2, and Lactobacillus fermentum under optimal conditions was four times higher than under optimal conditions [20]. This study was the first to report microbial production of vitamin B_2 from native and traditional cheese by probiotics from the Markazi Province. Vitamin B_2 was produced in varying amounts by the probiotic bacteria identified in this study, and the Lactobacillus fermentum strain SK152 isolate produced the highest amount of vitamin B_2 as 285.8 μg ml^{-1}.

CONCLUSIONS

Given the ability of Lactobacilli bacteria to produce important vitamin B group like B_2, the production of these vitamins and the mass production of these valuable compounds by these bacteria looks promising in the future. The results of the above studies on Lactobacillus, which show particularly high vitamin B_2 production, may be an effective idea for producing probiotic products using natural strains.

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