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Traditionally Fermented Foods as Source of Vitamin B12 Producing LAB

Lonkar Payal, Karmakar Payel and Walhe Rajan*

MES Abasaheb Garware College, affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.
*Corresponding Author's address: Dept. of Microbiology, MES Abasaheb Garware College Pune, Maharashtra, India. E mail: rawalhe@rediffmail.com

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ABSTRACT

Fermented foods are known to impose several health benefits to the human community, one of which is a source of bioactive compounds like vitamins due to microbial activities. Certain groups of lactic acid bacteria are reported to be capable of synthesis of B groups of vitamins. As vitamins present in food are easily destroyed during food processing and as humans are unable to synthesize most of the vitamins, deficiency of vitamins is a common problem in many individuals. Thus, including fermented food in diet can reduce the risk of vitamin deficiencies and other problems. In present study, a variety of traditionally fermented foods from different geographical areas and ethnic practices were prepared, and used as a source for isolation of lactic acid bacteria. The isolates were characterized for primary probiotic properties. The short-listed isolates were evaluated for vitamin B12 production ability. Vitamin B12 bioassay revealed thirteen isolates to be Vitamin B12 producers in the range of 0.6-1.9 ng/ml. Thus, traditionally fermented foods can be sources for lactic acid bacteria having vitamin B_{12} production ability with probiotic potential.

Key words: Fermented foods, Vitamin B12, Lactic acid bacteria, Probiotics.

INTRODUCTION

Fermented foods can generally be defined as "foods made through desired microbial growth and enzymatic conversions of food components" (Macro *et al*., 2021). Any food can be treated as a fermented food if one or more of its components are used by micro-organisms to produce a final product that is considerably altered and acceptable for human use (Kanwar *et al.,* 2007). Every fermented food is connected with a distinctive group of microbiota, which enhances the level of proteins, vitamins, essential amino acids and fatty acids (Jeyaram *et al*., 2009). Many fermented foods have shown the presence of a group of Lactic Acid Bacteria (LAB), mostly

species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weisella*, etc. (Tamang *et al.*, 2016). Micro-organisms use their metabolic pathways to derive energy from the compounds present in the fermented foods, during which they are known to produce certain bioactive components like lactic acid, short chain fatty acids (SCFA) and vitamins (Macro *et al.,* 2021). Asian traditional fermented foods are usually fermented by LAB including *Lactobacillus plantarum*, *L. brevis*, *L. fermentum*, *L. pentosus*, *L. casei*, *L. fallax*, *L. kimchi*, *Leuconosto cmesenteroides*, *Weissela confuse*, *W. koreenis, W. cibaria* and *Pediococcus pentosus* (Swain *et al*., 2014).

It is a well known fact that recently, people have adapted modern eating lifestyles which continuously contribute to our current health issues (McClements 2020). Modern food culture may lack the essential macro and micronutrients that are supposed to be present in the food to nourish our body. Such unhealthy diets are low in whole grains, fruits and vegetables, and high in sugar, salt, saturated fat and ultra-processed foods, proving to be a main threat factor for reduced health results and food fortification programs have eliminated vitamin deficiency problems (Mafra *et al.,* 2021, Tyler *et al.,* 2019). Besides this, vitamin B12 deficiency is a common disorder observed in vegan people as animal-derived products are the main source of vitamin B12. Studies have shown the ability of LAB to produce different types of metabolites that are together termed as 'neutraceuticals', and that includes B group of vitamins like riboflavin (B2), folic acid (B11) and cobalamine (B12) (Sathyanarayanan *et al.,* 2010).

In different fermented milk products like curd, yoghurt, cultured butter milks, and other fermented foods, biosynthesis and release of vitamin have been reported in successful support of LAB fermentation (LeBlanc at al 2007). Some of the people show lactose intolerance, thus the present study was focused on fermented cereal based products, fermented pulse based products and fermented vegetable based products i.e. non-dairy fermented products may be helpful with the context.

In the present work variety of traditionally fermented foods were used to isolate LAB with primary probiotic properties and studied for their ability to produce vitamin B12.

MATERIALS AND METHODS

Sample preparation:

Variety of traditionally fermented food samples were prepared. Following were the food/batter prepared and used for isolation of Lactic Acid Bacteria.

1. *Chik* (Wheat water) (Prepared using traditional methods): Ingredients: Wheat grains, water
Form of sample: Semi-solid.
Procedure: Wheat grains soaked in clean, warm water in a packed vessel, grains allowed to swell and grinded. Fermentation time:4-6 days.

2. Fermented ginger (Ginger ale): Ingredients: Ginger roots, sugar, water, vinegar, lemon juice (Prepared using traditional methods).Form of sample: Liquid.
Procedure: Ginger root soaked in sugar water, sugar was added daily, after a month of incubation, ginger is strained, vinegar, lemon juice added to it and stored. Fermentation time: 30 days

3. Kimchi (Link 1): Ingredients: Radish, cabbage. Form of sample: Solid.
Procedure: Cabbage is kept in saline water until all water content is released, washed, garlic, ginger, radish and spices are mixed, submerged in its own liquid and stored. Fermentation time:2-5 days.

4. Kanji (Link 2): Ingredients: Beetroot, carrot. Form of sample: Liquid
Procedure: Sliced carrot, beet and crushed mustard seeds combined in a glass jar, allowed to stand in a sunny spot, incubated until tangy flavor develops. Fermentation time:7 days

5. Mangalore buns (Link 3): Ingredients: Any or multi-purpose flour, ripened banana, sugar, yoghurt, oil. Form of sample: Solid.
Procedure: Ripened banana is smashed and mixed with sugar, yoghurt and flour, knead until smooth dough, kept covered for incubation and deep fried. Fermentation time:8 hours.

6. *Panta bhath* (*Poita bhath*) (Goswami *et al.,* 2016): Ingredients: Cooked rice, potato, onion, green chili, lime. Form of sample: Semi-solid.
Procedure: Overcooked rice stored in a closed jar overnight, mixed with smashed potatoes, green chili, salt and lime. Fermentation time: 12 hours.

7. *Sel roti* (Ashaolu and Reale, 2020): Ingredients: Rice flour banana, honey, sugar, ghee and spices. Form of sample: Solid.
Procedure: Banana, honey, ghee, spices and sugar mixed in the smooth paste of rice flour, incubated, squeezed, rings are made and deep fried. Fermentation time: 24 hours.

8. *Chakulipitha* (*Chakuli*) (Ray *et al.,* 2016): Ingredients: White lentils, rice, salt and water. Form of sample: Solid. Procedure: Soaked white lentils and rice are grind to make paste, salt is added, incubated and fried. Fermentation time: 6 hours.

Isolation, cultivation, media and growth conditions:
Samples were suspended in sterile saline, if necessary to dilute, vortexed and streaked on de Man Rogosa and Sharpe (MRS) agar (HiMedia), plates were incubated at 37 C for 24 to 48h under microaerophilic conditions. The isolates obtained were morphotyped and subjected to further study.

Hemolytic activity testing:
The hemolytic property of the isolates was determined by hemolysis test as described by Hosseini *et al.,* 2009 with slight change. The isolates were spot inoculated on blood agar plates (MRS agar + 5% blood), incubated at 37 C for 24-48h under microaerophilic conditions. The isolates showing no hemolytic property were selected for further studies.

Acid tolerance test:
Saline washed bacterial cells were resuspended in sterile Glycine-HCl buffer (pH 2.0) and sterile sodium citrate buffer (pH 3.0 and pH 4.0), incubated at 37 C for variable time intervals (60, 90, 120 minutes), followed by centrifugation (Singhal *et al.,* 2010). The bacterial cells were washed twice with sterile saline. Sterile MRS medium broth with 0.005 % bromo cresol purple was added in microfuge and the cultures were incubated at 37 C for 24-48h under microaerobic condition. The acid tolerant isolates were selected for further studies.

Bile tolerance test:
The selected isolates were inoculated in the sterile MRS broth containing 0.1%, 0.2% and 0.3% sodium taurocholate and incubated at 37 C for 24-48h under microaerobic conditions (Singhal 2010). The results were noted by comparing the test microfuge for turbidity with respect to bile control.

Biochemical characterization:
The short-listed isolates were further characterized to determine the mode of fermentation they follow (homo or hetero-fermentative) as well as checked for the production of enzymes like catalase and oxidase by them (Cruikshank 1975).

Screening of vitamin B12 producers:
The selected isolates were screened for their ability to synthesize vitamin B12 using sterile vitamin B12-free medium (Masuda et 2012). Isolates were streaked on the vitamin B12 free media and incubated at 37 C for 24-48h under microaerophilic conditions. Isolates showing growth were selected for the quantitative assay.

Vitamin B12 microbiological assay:
The standard assay was carried out as per Madhu et al., 2009 with some changes in the vitamin B12 assay media (HiMedia) using vitamin B12 standard (Cyanocobal-amin) and the auxotroph *E.coli* Davis A mutant strain 113-3D Culture was used.

i) Preparation of cell extract:
The selected isolates were grown for 24-48 h, centrifuged at 10,000 rpm for about 5 minutes to separate the cell mass. The cell mass was ice-chilled with sterile distilled water and vortexed. The procedure was repeated twice. Further, sterile 0.01% Tween 80 was added to this slurry followed by deep freezing (-20 C) and thawing for 2 hours. After overnight freezing, the slurry was thawed at 37 C and the lysed cell extract so formed was used as a source of vitamin B12.

ii) Vitamin B12 bioassay:
The sterile vitamin B12 assay medium agar was seeded with the suspension of *E.coli* Davis A mutant strain 113-3D and poured onto sterile petriplates. The dead cell slurry (25 µL) was added to the wells made on the bioassay media in triplicates kept for pre-diffusion at 25 C for about 20 minutes, and then kept for overnight incubation at 37 C. The plates were observed for zone of exhibition around the wells. The assay was conducted in triplicates and average of three was considered for calculations with the help equation from standard graph.

Antibiotic resistance/sensitivity determination:
Resistance pattern of some commonly used antibiotics was done for the selected isolates using polydisc method (Tambekar and Bhutada, 2010). The multiple drugs disc (Dodeca Universal- II [HiMedia]) was kept and pressed slightly on the sterile MRS agar media surface inoculated with the bacterial isolates. The plates were observed for the presence or absence of a zone of inhibition.

RESULTS AND DISCUSSION

The present study focuses on the isolation of lactic acid bacteria with primary probiotic properties. The isolates were from the traditionally fermented foods, and further screened the selected isolates for vitamin production.

Initially, total 40 isolates were obtained from Indian traditionally fermented foods such as *chik*, ginger ale, fermented aloe vera, kimchi, kanji, Mangalore buns, *pantabhath*, *Sel roti* and *chakolipitha* which were prepared using ethnic practices. All the isolates were morphologically characterized as Gram positive organisms (results not shown here). Out of 40 isolates 3 were found to be yeast. Present study was focused mainly on lactic acid microflora present in fermented foods, the 3 yeast isolates were excluded from the study.

The total of 37 isolates was further short-listed according to their primary probiotic potential. All the isolates obtained were tested for hemolysis on the basis of ability to lyse red blood cells. Out of 37 isolates, 7 isolates were observed to be hemolytic and 30 isolates were safe showing no hemolysis on blood agar (results not shown). The isolates that were non-hemolytic were selected for further studies.

The 30 short-listed isolates were further tested for the other probiotic functionality testing such as acid and bile tolerance (Fig 1a) and antibiotic susceptibility testing.

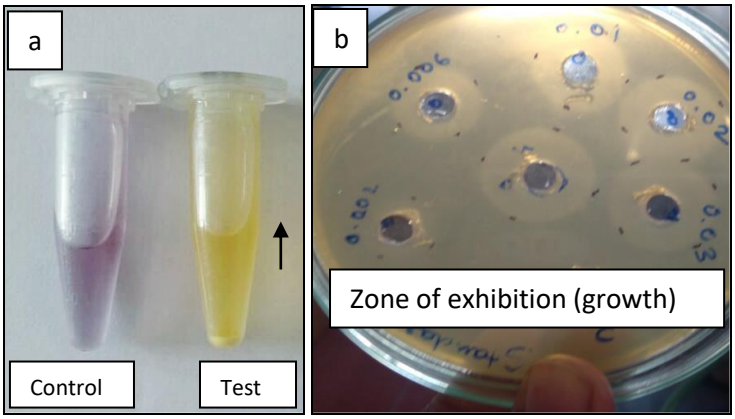

Fig 1. a) Acid and bile tolerance b) Vitamin B12 bioassay zone of growth

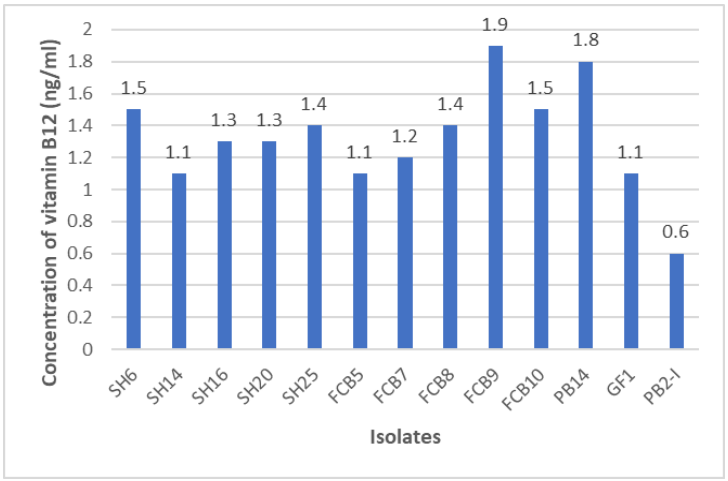

Fig 2. Vitamin B12 produced by the isolates

Table 1: Results of Antibiotic susceptibility test

Antibiotic	SH6	SH14	SH16	SH20	SH25	FCB5	FCB8	FCB9	FCB10	PB14
Amikacin	R	R	R	R	S	R	S	S	R	R
Co-Trimoxazole	R	R	R	R	R	R	R	R	R	R
Colistin	R	R	R	R	R	R	R	R	R	R
Augmentin	R	R	R	R	R	R	R	R	R	R
Netilin	S	S	S	S	S	S	S	S	S	S
Norfloxacin	R	R	R	R	R	R	S	S	R	R
Ceftriaxone	R	R	R	R	R	R	R	R	S	S
Ciprofloxacin	R	R	R	R	R	R	R	S	R	R
Cephotaxime	R	R	R	R	R	S	R	R	S	R
Gentamicin	R	S	S	S	S	R	S	S	R	S
Furazolidine	R	R	R	R	R	R	R	R	R	R
Amoxycillin	R	R	R	R	R	R	R	R	R	R

It was found that all the 30 isolates tested were tolerating acid for upto 120 minutes. This indicates that these organisms that are present in food will not be killed due to high acidity during their passage through the stomach.

It was noted that, out of 30 isolates tested, 16 survived 0.2% and 9 isolates namely GF1, AV2, FCB, FCB9, MAB2, PB2-I, PB14, SH6 and SH25 survived 0.3% of bile. The pH in the small intestine is about 0.2 (Faye *et al.,* 2011). This observation suggests that these tolerant strains are not being slaughtered in the intestine and thus survive to colonize the gut. Ability of the organisms to colonize in the gut can be determined by another probiotic property that is adherence to the epidermal cell line of the gut. This property of the isolate is not studied here, but the fact that these isolates are surviving in the presence of such high concentration of bile, increases their possibility of colonizing in the intestinal lining.

Isolates were tested for catalase (and oxidase) production to check whether they fall in the group of lactic acid bacteria. LAB are known to be negative for catalase and gram positive (Masuda *et al.,* 2012; Ray *et al.,* 2016). All the isolates tested for these biochemical tests showed negative catalase and oxidase tests except MAB2 (results not shown). Hence, it was eliminated for further studies. The biochemical tests performed were homo and heterofermentative tests to check the mode of fermentation (results not shown here). It was observed that the isolates PB14, SH14 and SH25 displayed hetero-fermentative mode of fermentation, that is they produced acid as well as gas during fermentation process. Although, this is not a desirable character as these organisms may cause bloating, the possibility that they can produce vitamin B12 in the food cannot be denied. Thus, even the hetero-fermenters were included in the study.

The main focus of the present study is to investigate traditionally fermented foods for vitamin B12 production of inhabiting LAB. Isolates were first studied qualitatively to check vitamin B12 producing ability. It was observed that total 13 isolates were vitamin B12 producers. Further, these isolates were studied for quantitative analysis of the vitamin B12 (Fig. 1b and Fig.2). The results demonstrated that all the 13 isolates produced vitamin B12 in the range of 0.6-1.9 ng/ml. It was found that the isolate FCB9 was the highest producer of vitamin B12 having concentration of 1.9 ng / ml. It has been reported previously that *Lactobacillus coryniformis* produced 1.8 µg/L and *L. plantarum* produced 2.0 µg/L. Both

strains were isolated from Japanese pickle (Masuda 2012).

Another report displayed that the strain *Lactobacillus reuteri* CRL1098, isolated from sourdough, also produced cobalamin (Taranto 2003). This strain has been reported to have all the genes necessary for cobalamin synthesis and thus attempts have been made in enhancing the production level of the vitamin B12 by metabolic engineering strategies. In our study, isolates are from indigenous food having advantage of better survival and benefits in the local population.

Another important desirable characteristic is resistance to the common antibiotics. For this characteristic study, 10 isolates showing highest vitamin B12 producing ability were selected. It was observed that all of the 10 isolates were resistant to the antibiotics Co-trimoxazole, Colistin, Augmentin, Furazolidine and Amoxycillin, whereas all the 10 isolates were found to be susceptible to Netilin (Table 1). Variable results were also observed such that all the isolates except SH6, FCB5 and FCB10 were susceptible to Gentamycin. Only SH25, FCB8 and FCB9 were susceptible to Amikacin and others were observed to be resistant.

CONCLUSIONS

Preparing fermented foods using traditional techniques and its consumption is a common practice in households. Its nutritive value is well known because of the process and quality of ingredients used in preparing it. Besides, the current study has proved that the microflora achieving the fermentation has an ability to reside in the human gut and produce B12 vitamin. Generally, animal-based products are the only source of vitamin B12. This may lead to deficiency disorders in the people preferring vegan diet. Along with this, many people are lactose intolerant, failing to take benefits of milk-based fermented foods. Thus, non-dairy fermented foods can gain popularity in near future as an alternative to vitamin supplements.

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Conflicts of Interest: The authors declare no conflict of interest.

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Link 2. kanji- https://myheartbeets.com/kanji-indian-probiotic-drink/

Link 3. Mangalore buns- https://hebbarskitchen.com/banana-buns-recipe-mangalore-buns/.