Vitamin B_{12} production and cholesterol reduction activity of *Pediococcus pentosaceus* isolates from fermented Indian foods

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Abstract

Indian food culture is rich with traditional fermentation practices. Fermentation enhances digestibility, flavor and aroma of food leading to enrichment of food substrates with protein, amino acids, vitamins etc. Vitamins are micronutrients that are essential for the metabolism of all living organisms. A deficiency of vitamin B_{12} is a potential public health problem. Atherosclerosis is identified as one of the causes in cardiovascular disease (CVD) and high serum cholesterol levels contribute to risk factors for atherosclerosis. Lactic acid bacteria (LAB) isolated from fermented foods has been reported to have vitamin B_{12} producing and cholesterol-lowering ability.

We report here two isolate strains- G11 and AP3 of Pediococcus pentosaceus from fermented Indian foods possessing vitamin B_{12} production and cholesterol reduction activities. Cell extracts of isolates were subjected to bioassay for vitamin B_{12}. Isolate AP3 produced 0.8 ng/ml vitamin B_{12}. We observed no significant increase in vitamin B_{12} production on addition of glycerol to the growth medium. These isolates were screened for Bile Salt Hydrolase (BSH) activity. Quantification of cholesterol reduction activity was done by an enzymatic method; AP3 and G11 reduced cholesterol in the medium by 50% and 40% respectively. Thus, these isolates can be considered as beneficial dietary supplements for functional foods.

Keywords: Fermented Food, *Pediococcus pentosaceus*, Vitamin B_{12}, Cholesterol Reduction.

Introduction

Scientific study is gaining attention towards fermented food as a dietary source of live microorganisms. This popularity of fermented food is because of their properties like increased shelf life, functionality, nutritional value due to variety of bioactive molecules and their other health beneficial properties including lowering the risk of cardiovascular and other clinical conditions[15]. Among bacteria associated with fermented foods are Lactic Acid Bacteria (LAB), mostly species of *Lactobacillus, Pediococcus, Weissella* etc.

Fermented foods have unique functional properties adding some health benefits to consumers due to presence of functional microorganisms with probiotic properties[18]. In the recent past, focus of scientific investigation on food has moved from preliminary role of food as a source of energy and body forming substances to fine action on human health; as a result a new term coined as functional food has been appended to it[6].

Vitamin B_{12} (cyanocobalmine) is an essential human nutrient. Its deficiency is affecting million of people and has became a worldwide health issue[2]. The deficiency may lead to anemia, neuropathy, hyperhomocysteinaemia and other problems. Vitamin B_{12} can be synthesized only by bacterial cells. Its recommended humans intake depends on age and other physiological status. The optimal daily intake dosage for adult is 2.4 µg[13]. LAB isolated from traditionally fermented foods are reported to produce Vitamin B_{12}.[11]

High serum cholesterol is a risk factor for coronary heart disease (CHD) and atherosclerosis[4]. Increased serum cholesterol concentration could be controlled by dietary intervention, therapeutics etc. However, cholesterol lowering drugs are associated with many side effects[3]. Therefore, there is need of alternative approach to deal with this problem. Cholesterol lowering probiotics strains are reported which make it through several mechanisms like; bile salt hydrolase (BSH) activity, bile salt deconjugation abilty, cholesterol assimilations etc. Wang et al[22] have shown LAB isolated from pickle mustard green with notable cholesterol reducing activity.

There are plenty of evidences indicating that LAB plays significant role in human health, also LABs are generally regarded as safe (GRAS) [24]. They have not only value in food industry but find equal importance in pharmaceutical industries. Traditional fermented Indian foods are known as source of LAB /probiotic bacteria and these foods include Idli, dosa, Dhokala, pickles, appam etc.[8,9,17] In the present study we have evaluated vitamin B_{12} production and cholesterol reduction ability of *Pediococcus pentosaceus* isolated from fermented Indian foods and their identification confirmed by Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS.

Material and Methods

Bacterial isolates, culture media and growth conditions: LAB isolates were short listed from pool of lactic cultures obtained from traditionally fermented Indian foods and studied for potential probiotic properties[21]. These have been identified by 16S r RNA gene sequence analysis as: G11: *Pediococcus pentosaceus*, AP3: *Pediococcus pentosaceus* and were deposited in National Centre for Microbial Resource (NMCR), Pune (accession number MCC 3768 and MCC 3769 respectively). These cultures were grown in de

Man Rogosa and Sharpe (MRS) medium (HiMedia) under microaerophilic conditions and used here for further study viz. MALDI-TOF-MS analysis for identification confirmation, vitamin B_{12} production and cholesterol reduction activity.

MALDI-TOF-MS analysis: MALDI-TOF-MS (Matrix-assisted laser desorption ionization time-of- flight mass spectrometry) technique was used for identification of bacteria using ethanol-formic acid extraction method[14]. The identification results of this study were expressed by BioTyper log (score)[5].

Vitamin B_{12} production testing: Prior to testing for vitamin B_{12} production, the LAB were screened for their ability to synthesize vitamin B_{12} by growing them on vitamin B_{12} free medium[11]. The production of vitamin B_{12} was tested in lysed cell extract. The bioassay of vitamin B_{12} was performed on plates[10, 19]. For the bioassay, vitamin B_{12} requiring auxotroph; *E. coli* Davis A mutant was procured from NCIM, Pune and maintained in laboratory. The standard of vitamin B_{12} for assay was the injection Optineuron 3 ml containing 1000µg of cyanocobalamine (LUPIN Ltd.) and used for construction of standard graph in the vitamin B_{12} bioassay medium (HiMedia).

Preparation of cell extract: Culture isolates were grown for 48 h and counts determined in triplicate (10^{10} CFU/ml). Cells were separated by centrifugation and to the cell pellet, ice chilled sterile distilled water added, mixed by vortex and centrifuged again. 180 µl of sterile distilled water was added to the pellet and mixed to make slurry. To this slurry, 20 µl sterile 0.01% Tween 80 (Merck) was added and mixed. Slurry was allowed freeze at -20 C, for 2 h and then thawed at 37^0C by mixing. Slurry was again freezed at -20^0C overnight. After overnight freezing, the slurry was thawed and this lysed cell extract (dead dell slurry) was used as a source of vitamin B_{12} from the isolates.

Bioassay:100 ml vitamin B_{12} bioassay medium was seeded with 500 µl saline suspension of *E.coli* Davis A strain (0.5 OD at 615 nm) and poured (bioassay plates) and allowed to solidify. After solidification, wells (diameter 6mm) were prepared and 25 µl of dead cell slurry (cell extract) added to the wells. Along with the dead cell slurry, vitamin B_{12} standard of different dilutions were also added to different wells. Control (sterile distilled water) was added to wells. After pre-diffusion for about 20 minutes, plates were incubated at 37C for overnight.

After incubation, exhibition zone of growth of *E. coli* was measured with the help of digital Vernier caliper and results were recorded as an average of three. Standard graph for vitamin B_{12} was constructed (also in triplicate). From the standard graph, vitamin B_{12} content in dead cell extract was determined. Effect of glycerol on vitamin B_{12} production was tested by incorporating 0.5% glycerol in the growth medium[16].

Cholesterol reduction ability testing

BSH activity: The isolates were screened semi quantitatively for bile salt hydrolase (BSH) activity as described by Mathara et al[12]. The assay was performed in triplicate. In brief the protocol was: The LAB were inoculated in MRS broth and incubated for 24 h. 10 µl of the culture isolate (10^{10}CFU/ ml) inoculated onto BSH screening medium (MRS agar was supplemented with 0.5% bile salt and 0.037 % $CaCl_2$). Plates were incubated at 35^0C for 24 to 48 h. After the incubation, the precipitation zone diameter was measured and recorded as average of three.

Cholesterol reduction activity: Cholesterol reduction activity of the selected isolates was evaluated by growing the cultures in MRS broth containing cholesterol and then estimating the cholesterol by enzymatic method[23].

In brief, the protocol followed was: From the fresh growth of culture isolates growth in MRS, 50 µl was inoculated to 1000 µl MRS broth containing 200 µg/ml filter sterilized cholesterol from the kit (AUTOSPAN) .Sterile distilled water was used as control. The tubes were incubated for 24h. All the tubes were centrifuged to separate the cells. The cholesterol was estimated in the supernatant broth by enzymatic method using kit(AUTOSPAN). The cholesterol was expressed as cholesterol µg/ml as an average of three readings.

Results and Discussion

MALDI-TOF-MS is now used for identification of microorganisms. Gracia et al[5] have showed 100% congruency between 16S r RNA gene analysis and MALDI-TOF-MS in identification of *Lactobacillus spp*.

MALDI-TOF-MS, used for identification of microorganisms is a phenotype based method and utilizes the specific protein profiles[7]. It has advantages like high throughput, easy to perform and accurate and is used in gut microbiotas. The MALDI-TOF mass spectra measurements using Biotyper were obtained (Fig. 1). The strains showing ≥1.7 log value with strain in database were confirmed as the member of that genus and strains showing ≥2.0 log (2.3 to 3) values were confirmed as the member of that species. The log (scores) showed more than 2.3 for both cultures (Table 1), confirming the isolate G11 and AP3 as *Pediococcus pentosaceus* which goes parallel with 16S r RNA gene analysis done.

Both isolates were shown to produce vitamin B_{12} (Fig.2). Vitamin B_{12} produced was calculated from the straight line equation from standard graph. Isolate G 11, AP3 produced about 0.8 ng/ml vitamin B_{12}. Glycerol has been reported to exert regulating effect on vitamin B_{12} production.[16]

However, incorporation of glycerol (0.5%) in the growth medium did not result significant change in vitamin B_{12} production in our study by these LAB. Since these LAB were isolated from food and showed mucosal adherence

properties[20]; incorporation of these cultures for food fermentation can offer vitamin B_{12} to consumer.

The LAB cultures exhibited BSH activity as indicated by zone of precipitation (Fig. 3). It was seen that all LAB isolates reduced cholesterol in the broth (Fig. 4). Albano et al[1] also reported 45 to 55% reductions in cholesterol by LAB isolates from Italian dairy products. Our isolate from Indian fermented food AP3 has shown reduction up to 50% and G11 40% which are comparable. Thus, BSH activity and reduction of cholesterol by LAB isolates suggest its application in lowering serum cholesterol levels and thereby decrease the risk of CHD and atherosclerosis.

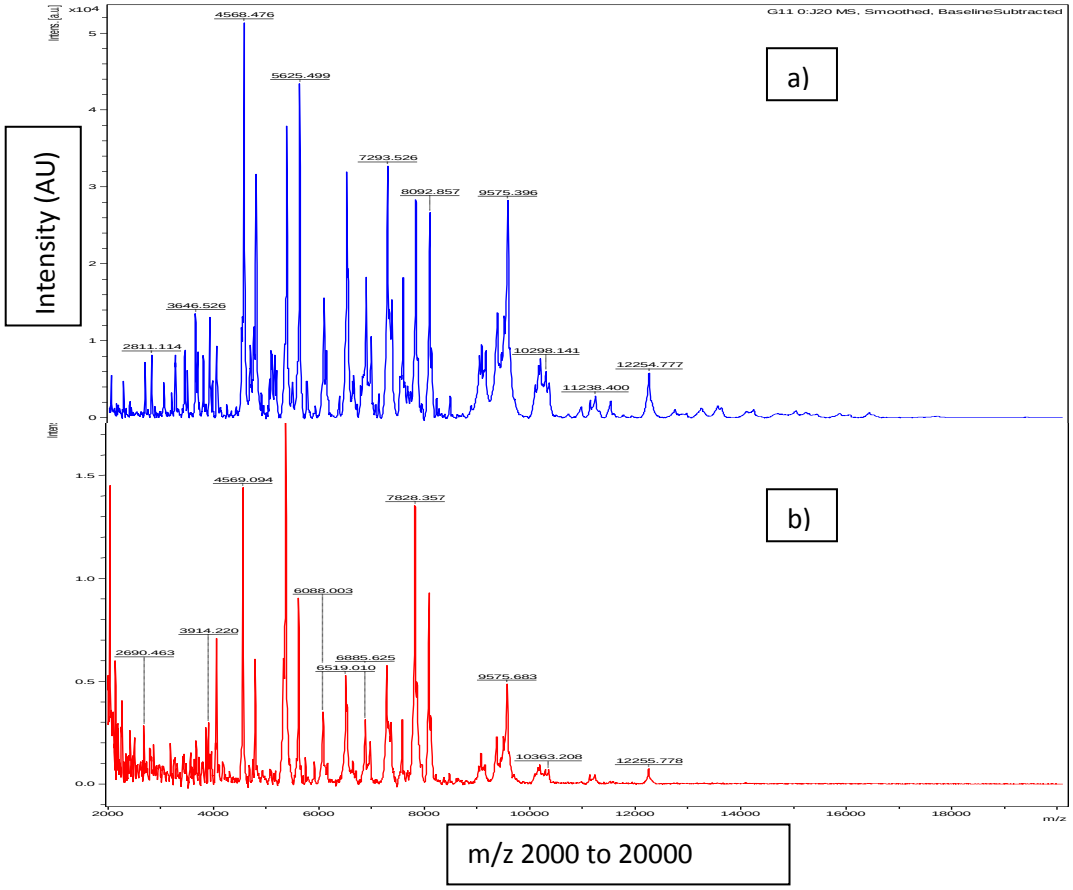

Fig. 1: MALDI-TOF-MS Spectra of a) G11 and b) AP3

Table 1
MALDI-TOF-MS identification chart

S.N.	Analyte	Identification	Score value
1	G11	*Pediococcus pentosaceus*	2.318
2	AP3	*Pediococcus pentosaceus*	2.352

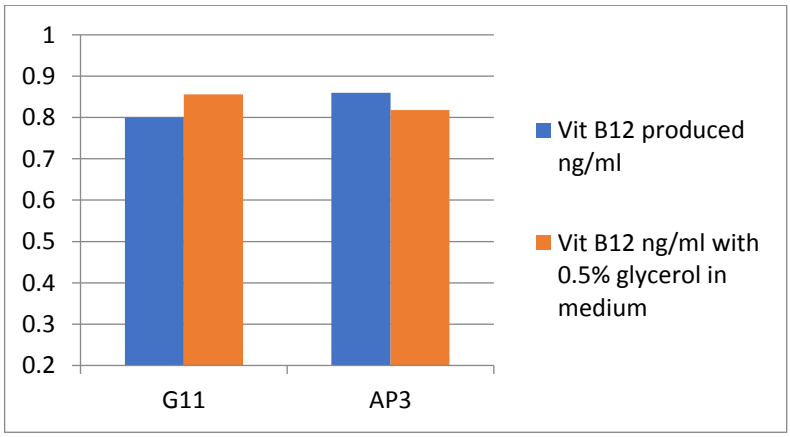

Fig. 2: Vitamin B_{12} produced by LAB cultures

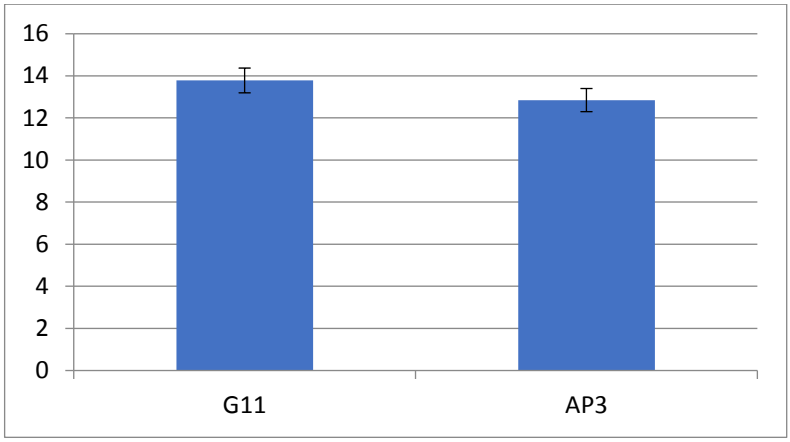

Fig. 3: BSH activity o LAB isolates (Precipitation zone diameters in mm)

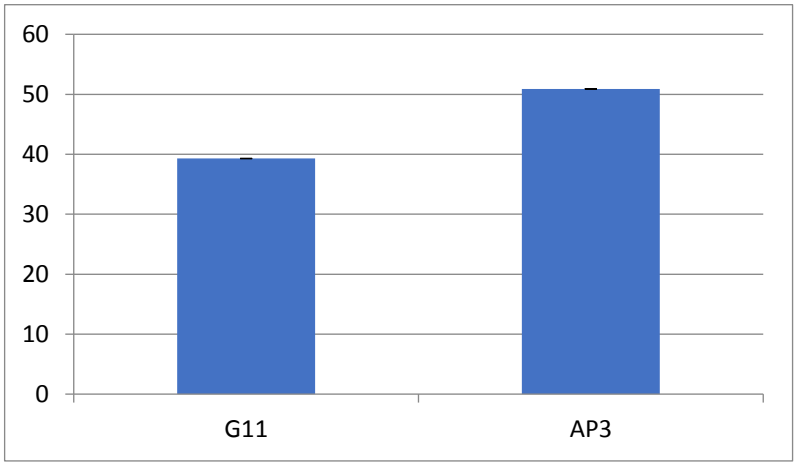

Fig. 4: Per cent Reduction of cholesterol by LAB isolates

Conclusion

The reconstitution of the normal composition of the intestinal flora by appropriately selected LAB may have the ameliorating effect on vitamin B_{12} deficiency associated disorders. The risk of CVD /CHD associated with the elevated cholesterols levels can be moderated by the selective LAB with cholesterol reducing properties. Our study indicates the potential application of the LAB with studied probiotic properties which are isolated from fermented Indian foods as beneficial effect in vitamin B_{12} deficiency and elevated cholesterols levels.

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References

1. Albano C., Morandi T., Silvetti M.C., Casiraghi F.M. and Brasca M., Lactic acid bacteria with cholesterol-lowering properties for dairy applications: In vitro and in situ activity, *J. Dairy Sci*, **101**, 10807- 10818 **(2018)**

2. Baltaci D., Deler M.H., Turker Y., Ermis F., Iliev D. and Velioglu U., Evaluation of serum Vitamin B12 level and related nutritional status among apparently healthy obese female individuals, *Niger J Clin Pract*, **20**, 99-105 **(2017)**

3. Bhat B. and Bajaj B.K., Multifarious cholesterol lowering potential of lactic acid bacteria equipped with desired probiotic functional attributes, *3 Biotech*, **10(200)**, 1-16 **(2020)**

4. El Helim M.A., Hashem S., Essam T. and Omar M., Cholesterol Lowering Capability of some Lactobacillus Strains and its Effect on Mice fed a High cholesterol diet, *Clinical Microbiology: Open Access*, **5(3)**, 1-4 **(2016)**

5. Garcia E.F., Luciano W.A., Xavier D.E., da Costa W.C.A., de Sousa Oliveira K., Franco O.L., de Morais Junior M.A., Lucena B.T.L., Picao R.C., Magnani M., Saarela M. and de Souza E.L., Identification of Lactic Acid Bacteria in Fruit Pulp Processing Byproducts and Potential Probiotic Properties of Selected Lactobacillus Strains, *Front. Microbiol*, **7(1371)**, 1-11 **(2016)**

6. Granato D., Branco G.F., Nazzaro F., Cruz A.G. and Faria J.A., Functional foods and nondairy probiotic food development: trends, concepts and products, *Comprehensive Reviews in Food Science and Food Safety*, **9(3)**, 292-302 **(2010)**

7. Huang C. and Huang L., Rapid species- and subspecies-specific level classification and identification of *Lactobacillus casei* group

members using MALDI Bio typer combined with Clin Pro Tools, *J. Dairy Sci*, **101**, 979–991 **(2018)**

8. Iyer B.K., Singhal R.S. and Ananthanarayan L., Characherization and *in vitro* probiotic evaluation of lactic acid bacteria isolated from *Idli* batter, *Journal of Food Science and Technology*, **50(6)**, 114-21 **(2013)**

9. Jamuna M. and Jeevaratnam K., Isolation and characterization of lactobacilli from some traditional fermented foods and evaluation of the bacteriocins, *The Journal of General and Applied Microbiology*, **50(2)**, 79-90 **(2004)**

10. Madhu A.N., Giribhattanavar P., Narayam M.S. and Prapulla S.G., Probiotic lactic acid bacterium from *Kanjika* as a potential source of vitamin B12: evidence from LC-MS, immunological and microbiological techniques, *Biotechnology Letters*, **32,** 503-6 **(2010)**

11. Masuda M., Ide M., Utsumi H., Niiro T., Shimamura Y. and Murata M., Production potency of folate, vitamin B12 andthiamine by lactic acid bacteria isolated from japanese pickles, *Bioscience Biotechnology and Biochemistry*, **76(11)**, 2061-7 **(2012)**

12. Mathara J.M., Schillinger U., Guigas C., Franz C., Kutima P.M., Mbugua S.K., Shin H.K. and Holzapfel W.H., Functional characteristics of *Lactobacillus spp*. from traditional *Maasai* fermented milk products in Kenya, *International Journal of Food Microbiology*, **126**, 57-64 **(2008)**

13. Piwowarek K., Lipinska E., Hac-Szymanczuk E., Kieliszek M. and Scibisz I., Propionibacterium spp.- source of propionic acid, vitamin B12 andother metabolites important for the industry, *Appl Microbiol Biotechnol*, **102**, 515-538 **(2018)**

14. Rahi P., Prakash O. and Shouche Y.S., Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass-Spectrometry (MALDI-TOF MS) Based Microbial Identifications: Challenges and Scopes for Microbial Ecologists, *Front. Microbiol*, **7(1359)**, 1-12 **(2016)**

15. Rezac S., Kok C.R., Heemann M. and Hutkins R., Fermented foods as dietary source of live organisms, *Frontiers in Microiology*, **9(1785)**, 1-29 **(2018)**

16. Santos F., Spinler J.K., Saulnier D.M., Molenaar D., Teusink B., de Vos Versalovic J. and Hugenholtz J., Functional identificationin *Lactobacillus reuteri* of a PocR-like transcription factor regulating glycerol utilization and vitamin B12 synthesis, *Microbial Cell Factories*, **10(55)**, 1-11 **(2011)**

17. Satish Kumar R., Kanmani P., Yuvraj N., Paari K.A., Pattukumar V. and Arul V., Traditional Indian fermented foods; a rich source of lactic acid bacteria, *International Journal of food sciences and Nutrition*, **64(4)**, 415-28 **(2013)**

18. Tamang J.P., Watanabe K. and Holzapfel W.H., Diversity of Microorganisms in Global Fermented Foods and Beverages, *Front. Microbiol*, **7(377)**, 1-28 **(2016)**

19. Taranto M.P., Vera J.L., Hugenholtz J., De Valdez G.F. and Sesma F., *Lactobacillus reuteri* CRL1098 produces cobalamin, *Journal of Bacteriology*, **185**, 5643-7 **(2003)**

20. Walhe R.A., Patole M.S. and Diwanay S.S., Adhesion inhibition of *Salmonella enterica* subspecies *typhimurium* to HT-29 cells by LAB isolates from traditionally fermented Indian foods, *Int. Res. Journal of Science & Engineering*, **6(2)**, 31-34 **(2018)**

21. Walhe R.A., Patole M.S. and Diwanay S.S., Some beneficial properties of bacterial isolates from traditionally fermented Indian foods, *Bionano Frontier*, **10(1)**, 120-126 **(2017)**

22. Wang S.C., Chang C.K., Chan S.C., Shieh J.S., Chiu C.K. and Duh P.D., Effects of lactic acid bacteria isolated from fermented mustard on lowering cholesterol, *Asian Pacific Journal of Tropical Biomedicine*, **4(7)**, 523-8 **(2014)**

23. Wu C.C., Weng W.L., Lai W.L., Tsai H.P., Liu W.H., Lee M.H. and Tsai Y.C., Effect of *Lactobacillus plantarum* strain K21 on high-fat diet-fed obese mice, *Evidence-Based Complementary and Alternative Medicine*, **2015,** 1-9 **(2015)**

24. Xu Y., Cui Y., Yue F., Liu L., Shan Y., Liu B., Zhou Y. and Lu X., Exopolysaccharides produced by lactic acid bacteria and *Bifidobacteria*: Structures, physiochemical functions and applications in the food industry, *Food Hydrocolloids*, **94**, 475–499 **(2019)**.

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