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Vitamin B_{12} enriched milk based nutraceutical production using *Propionibacterium freudenreichii*

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Abstract

A *Propionibacterium freudenreichii* strain that was well characterized for its probiotic characteristics like pH, bile salt tolerances, sensitivity to commonly used antibiotics was employed as a starter culture to develop vitamin B_{12} enriched nutraceutical product. The experiment was designed using five treatments with starter inocula as home-made curd (T_1), *Propionibacterium freudenreichii* (T_2), *Lactobacillus acidophilus* (T_3), *Propionibacterium freudenreichii* + *Lactobacillus acidophilus* (T_4) and yoghurt (T_5). Milk was fermented at 37 °C for 8 h using these starter cultures. The developed product after fermentation was analyzed for vitamin B_{12} content, physico-chemical properties and was subjected to sensory evaluation. Treatment T_2 with inoculum *Propionibacterium freudenreichii* was found to produce the significantly highest vitamin B_{12} (10.37 µg $100mL^{-1}$) along with the best score (8.15) of acceptance for sensory evaluation parameters. The second best sensory score was given to treatment T_5 (7.9) while the second highest vitamin B_{12} production was found in treatment T_4 (8.34 µg $100mL^{-1}$). One serving of the product (10.37µg$100mL^{-1}$) provides vitamin B_{12} more than recommended daily allowance (2.4 µg).

Keywords: Nutraceutical beverage, propionibacterium freudenreichii, sensory evaluation, vitamin B_{12} and yoghurt

1. Introduction

Vitamin B_{12} is one of the water soluble B-complex vitamins and it is commonly called as "Cobalamin". It acts as a cofactor for many different enzymes in the human body and plays numerous roles in proper functioning of human metabolism (Ball, 2006) [2]. Rich sources of cobalamin are meat, milk, fish, shell fish and eggs. None of the vegetarian foods are reported as sources of cobalamin, making the vegetarians and vegans deficient for cobalamin. Deficiency of Cobalamin leads to pernicious anaemia and severe deficiencies were associated with neurological damage of brain cells (Hoffbrand and Provan, 1997) [7] however, a subnormal blood level of cobalamin was linked to reversible dementia (Tucker *et al.*, 1996) [25]. Cerebellar ataxia and leukoencephalopathy was also found to be associated with cobalamin deficiency (Morita *et al.*, 2003) [16]. The recent investigations with vitamin B_{12} deficiency were found to be associated manifestations (Sahu *et al.*, 2022) [21]. Vitamin B_{12} levels were found to be very low in HIV patients (Hepburn *et al.*, 2004) [9] while, their treatment with cobalamin demonstrated anti-retroviral activity (Weinberg *et al.*, 1995) [30]. The possibility of slow onset of AIDS in HIV patients was found to be associated with adequate vitamin B_{12} (Anonymous, 1998) [1]. Hence, development of cobalamin rich foods gets priority as a need based research.

Commercial production of cobalamin done by microbial synthesis, as chemical synthesis is not followed because of more than 30 steps are involved it, making the process complex and a costly affair. Many bacteria were screened for production of cobalamin through microbial synthesis, but the quantity of production varies among them (Perlman, 1959) [19]. *Propionibacteria* are identified to produce stable and higher quantities of cobalamin. *Propionibacteria* spp. that are capable of producing cobalamin are generally regarded as safe (GRAS) microorganisms and have the probiotic characteristics, making the *Propionibacterium* spp. better agents in synthesis of cobalamin rich foods.

Beneficial microorganisms in fermented foods have two major roles, microbiological property of fermenting the food by enhancing its shelf life and conferring health benefits by producing special metabolites into it. Propionic acid bacteria fall into this category by producing numerous human health beneficial metabolites (Ekinci and Gurel, 2008) [6]. Propionibacteria spp. are the best known starter cultures in the preparation of Swiss cheese, they are known for their characteristic eye formation in cheese due to production of CO_2 and also fruity and nutty flavors produced by propionate and acetate (Biede and Hammond, 1979) [3]. The Propionibacteria are employed in various dairy products and are considered as potential probiotic microorganisms (Thiel *et al*., 2004) [24]. *Propionibacterium freudenreichii* was found to ameliorate acute colitis in rat model (Ma *et al*., 2020) [15]. The Propionibacterium 702 strain was found to survive in gastrointestinal tract of male Wistar rat during transit without affecting the health (Huang *et al*., 2003) [10]. The probiotic potential of dairy propionibacteria was attributed due to production of vitamin B_{12}, propionic acid, bacteriocins, growth stimulation of other bacteria in gastrointestinal tract, survival during the process of digestion (Kaneko *et al.*, 1994; Perez-Chaia *et al.*, 1999; Holo *et al*., 2002; Hugenholtz *et al.*, 2002; Warminska Radyko *et al.*, 2002; Ekinci and Barefoot, 2006) [12, 18, 8, 11, 29, 5].

Milk is considered as a vegetarian food and forms the complete medium for many beneficial microorganisms. Hence, this property of milk was considered to develop a probiotic product of *Propionibacterium freudenreichii*. This experiment was aimed to develop vitamin B_{12} enriched milk based fermented product using *Propionibacterium freudenreichii*, that contains higher quantity of vitamin B_{12} than the local curds by using the standard checks such as yoghurt cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*).

2. Material and Methods

The fermentation experiment was conducted in the Department of Agricultural Microbiology, Gandhi Krishi Vigyan Kendra, University of Agricultural Sciences, Bengaluru. The material and methods used in the experiment are as follows

2.1 Starter cultures

Propionibacterium freudenreichii was isolated from cheese that was procured from the local market using Yeast Extract Lactone Agar Medium (YELA). It was characterized using 16 S rRNA sequencing (NCBI accession number: MT742831). The isolation, biochemical characterization, tolerance to pH and bile salts, sensitivity to commonly used antibiotics, bio-control potential against common human pathogens of *Propionibacterium freudenreichii* was discussed in the previous paper (Kumar *et al.,* 2020) [14]. *Lactobacillus acidophilus* (NCIM 2903) strain procured from the National Collection of Industrial Microorganisms (NCIM), Pune was used as a starter culture as one of the treatments and also as an adjunct culture along with *Propionibacterium freudenreichii* in another treatment. Yoghurt was procured from the local market was used as a starter inoculum (Comparative check). The *Propionibacterium freudenreichii* was maintained on Yeast extract lactone agar (YELA) (Van Niel, 1928) [26] and the *Lactobacillus acidophilus* was maintained on De Man, Rogosa and Sharpe's (MRS) agar medium (De Man *et al.,* 1960) [4].

2.2 Treatments

The experiment was designed with control (T_1), using local curd (homemade) as inoculum, *Propionibacterium freudenreichii* as starter culture (T_2), *Lactobacillus acidophilus* (T_3) a standard check. The fourth treatment (T_4) was a combination of T_2 and T_3, to find out the synergistic effect of *Propionibacterium freudenreichii* and *Lactobacillus acidophilus* in vitamin B_{12} production and sensory evaluation parameters. The fifth treatment (T_5) was a comparative check comprised of yoghurt cultures.

Table 1: Details of treatments

Sl. No.	Particulars	Details
1.	Design	Completely randomized design
2.	Replications	Three
3.	Treatments	T1 = Absolute control (Without microbial inoculants) T2 = *Propionibacterium freudenreichii* T3 = *Lactobacillus acidophilus* T4 = T2 + T3 T5 = Yoghurt isolates

2.3 Product development or curdling experiment

Homogenized cow milk procured from market was used in the experiment. Screw cap glass bottles (200 mL capacity) were used as fermentation jars in the experiment. Milk was heated to 90 °C for 20 minutes and was dispensed (100 mL) into glass bottles in a laminar air flow chamber. The overnight grown broth culture of the *Propionibacterium freudenreichii* isolate was centrifuged at 6,000 rpm for 10 min to obtain the cell pellet. The cell pellet was dispersed in the normal saline and plated on to YELA medium before inoculating to milk at the rate of 5% v/v. The other inocula of starter cultures were added as curd sample and yoghurt samples 5% (v/v). The inoculated glass bottles were incubated at 37 °C for eight hours to obtain the fermented product.

2.4 Preparation of vitamin B_{12} standards

Vitamin B_{12} standard reagents were prepared to develop a standard graph in HPLC for analysis of vitamin B_{12} in the product. All the standards were prepared in methanol solvent. HPLC grade vitamin B_{12} of Himedia was used for preparation of the standards. Initially 1 mg of standard vitamin B_{12} was dissolved in 5 mL of methanol and final volume was made to 10 mL to give a concentration of 1000 ppm. Other concentration of 100 ppm was prepared by taking 1 mL of solution from 1000 ppm and dissolving it in methanol and final volume was made to 10 mL. Similarly 10 ppm, 1 ppm and 0.5 ppm standards were prepared yielding five levels of standards *viz.* 0.5, 1.0, 10, 100 and 1000 ppm.

2.5 Sample extraction

Extraction of vitamin B_{12} was done by direct mixing of buffer solution (KCN-acetate) with pH-4.5 and product samples in the ratio of 10:4 (v/v) (Van Wyk and Britz, 2010 [27]). The whole setup, stored in darkness as vitamin B_{12} is photosensitive. The product-buffer solution was heated to 121 °C for 25 min and was allowed to cool. Later the solution was centrifuged at 10,000 rpm at 4 °CW and the resultant supernatant was collected and filter sterilized using membrane filter fitted in a syringe. The filtrate was stored under dark conditions and was used for HPLC analysis.

2.6 High Pressure Liquid Chromatography (HPLC)
The HPLC analysis was performed using I series Shimadzu system fitted with C18 column with reverse phase chromatography. The system was fitted with a volume adjustable auto-sampler. The vitamin B_{12} readings were taken at 361 nm wavelength, with methanol and water in the ratio of 30:70 as solvents.

Table 2: Functional standards adopted in HPLC analysis

Sl. No.	Parameter	Range
1.	Solvent system / Mobile phase	Methanol: Water 30:70
2.	Pressure variation	Isocratic
3.	Wavelength	361 nm
4.	Run time	4 min
5.	Retention time	2.9 min
6.	Column	C18
7.	Detector	UV detector
8.	Auto-sampler volume	5 µL

2.7 Titrable acidity
The product (15 mL) was taken in a conical flask, four drops of phenolphthalein indicator was added and titrated against 0.1 N NaOH. The appearance of light pink colour indicates the end of the reaction.
The titrable acidity was calculated using the formula

$$\text{Titratable acidity (\%)} = \frac{\text{Volume of titrant } \times \text{N} \times 90}{\text{Weight of sample } \times 1000} \times 100$$

Where, 90 = equivalent weight of lactic acid; N = normality of titrant

2.8 Total soluble solids
Total soluble solids in probiotic product were analysed using optical hand refractometer. A small homogenized drop of each sample was taken and put on the prism plane and viewed for brix value.

2.9 Microbial population in the product
The initial and final population of inoculated microbial strains in the probiotic beverage were enumerated by standard plate count method. YELA medium was used enumeration of *Propionibacterium freudenreichii* and MRS agar medium for *Lactobacillus acidophilus* and yoghurt cultures.

2.10 Sensory evaluation
A sensory evaluation sheet of nine point hedonic scale was developed for the product (Appendix-1). Since, the product was milk based and fermented most of the sensory parameters related to curd were adapted in evaluation. The other parameters related to the product were also analysed such as initial and final pH.

3. Results and discussion
The amount of vitamin B_{12} available under normal conditions in local curds is 0.4 µg per 100 mL, for a person to attain his recommended daily allowance (2.4 µg). Hence, there is a need to consume 600 mL of curd that would be a difficult task on daily basis to consume such large quantity. Hence, the present experiment was developed to include higher quantity of vitamin B_{12} into curd in order to meet RDA and to include the probiotic benefits of *Propionibacterium freudenreichii*. The results of fermentation experiment are presented below.

3.1 pH
The treatment T_2 product had pH (5.16) after the fermentation as *Propionibacterium freudenreichii* is a slow growing bacterium and the generation time is longer. The pH of T_5 treatment was found to be the lowest (4.56) after 8 h of fermentation, a two unit reduction of pH from the initial value was observed, this was in compliance with observations made by Rademaker *et al.*, 2006 [20].

3.2 Titrable acidity (%) and Total soluble solids (%)
The titrable acidity in product accounts for the acids released by the starter cultures. The highest (0.56%) and the lowest (0.45%) (Table No.3) titrable acidity values were recorded with T_3 and T_2 respectively. Similar values of titrable acidity in between 0.48% to 0.51% were found be Wardani *et al.*, 2017 [27], at 5% level inoculum with incubation temperature of 37 °C for 8h. The initial total soluble solids in the milk were found to be 12.6. The starter cultures utilize the total soluble solids present in milk and reduce them during curd formation. The highest total soluble solids reduction was found in treatment T_5 (10.4%).

Table 3: Physico-chemical properties of the product

Treatment No.	Starter cultures	pH		TSS (° Brix)		Titrable acidity (%)	
		Initial	Final	Initial	Final	Initial	Final
T_1	Home made curd	6.60	4.90^{ab}	12.6	11.4^{ab}	0.14	0.560^{a}
T_2	*Propionibacterium freudenreichii*	6.60	5.16^{a}	12.6	11.7^{a}	0.14	0.445^{c}
T_3	*Lactobacillus acidophilus*	6.60	4.49^{bc}	12.6	11.2^{bc}	0.14	0.561^{a}
T_4	$T_2 + T_3$	6.60	4.83^{cd}	12.6	10.9^{c}	0.14	0.460^{b}
T_5	*Lactobacillus bulgaricus + Streptococcus thermophilus*	6.60	4.56^{d}	12.6	10.4^{d}	0.14	0.559^{a}

3.3 Microbial population
After eight hours of incubation the highest microbial population (38.0×10^8 cfu mL^{-1}) (Table No. 4) was found in T_5 *i.e.* in yoghurt starter inoculated treatment. Since, the generation time of *Propionibacterium freudenreichii* was longer, a lower population (0.047×10^8 cfu mL^{-1}) growth was observed. Similar trend of lower population growth was exhibited by *P. freudenreichii* in cow's milk compared to *Lactobacillus plantarum* (Tarnaud *et al.*, 2020) [23].

Table 4: Microbial population and Vitamin B_{12} content in the product

Treatment No.	Starter cultures	Population (x 10^8 cfu mL^{-1})		Vitamin B_{12} (µg 100 mL^{-1})
		Initial population	Final population	
T_1	Homemade Curd	0.33	31.0	0.48^e
T_2	*Propionibacterium freudenreichii*	0.018	0.047	10.37^a
T_3	*Lactobacillus acidophilus*	0.32	26.0	1.41^d
T_4	$T_2 + T_3$	0.40	29.0	8.34^b
T_5	*Lactobacillus bulgaricus + Streptococcus theromophilus*	0.57	38.0	2.04^c

3.4 Vitamin B_{12}

The highest quantity of vitamin B_{12} was produced by T_2, inoculated with *Propionibacterium freudenreichii* (10.37 µg 100mL^{-1}) (Table No. 4). The lowest vitamin B_{12} quantity was produced in the treatment of T_1 receiving home-made curd inoculum (0.48 µg 100mL^{-1}). Ekinci and Gurel, 2008 [6], used *Propionibacterium freudenreichii* as adjunct culture in yoghurt preparation, in a similar way *Lactobacillus acidophilus* was used as an adjunct culture with *Propionibacterium freudenreichii* in anticipation of improving functional properties. The vitamin B_{12} production was not increased or enhanced in this treatment (T_4) (*Propionibacterium freudenreichii* + *Lactobacillus acidophilus*) compared to treatment T_2. This reduction in vitamin B_{12} may be attributed due to its utilization by *Lactobacillus acidophilus* for its growth as referred by Khattab *et al.*, 1980 [13]. The comparative check (T_5) had produced comparatively higher vitamin B_{12} (2.04 µg 100mL^{-1}) than control T_1 (0.48 µg 100mL^{-1}) and T_3 (1.41 µg 100mL^{-1}), this may be attributes to synthesis of vitamin B_{12} by *Lactobacillus bulgaricus* as reported by Shahani *et al.*, 1974 [22].

3.5 Sensory evaluation

An expert panel for sensory evaluation was done by a team of twelve members. To evaluate the product a nine point hedonic scale was used and the parameters used in the sensory evaluation were appearance, color, taste, flavor, firmness and overall acceptability. The highest score (8.15) (Table No. 5) was observed with T_2 (inoculated with the *Propionibacterium freudenreichii*) while the lowest was scored was by T_1 (Home-made curd inoculum).

Table 5: Sensory evaluation of the product

Treatment No.	Starter cultures	Appearance (9)	Colour (9)	Taste (9)	Firmness (9)	Flavour (9)	Overall Acceptability (9)
T_1	Home-made curd	7.6	7.4	6.9	7.0	7.2	7.15
T_2	*Propionibacterium freudenreichii*	8.1	8.3	8.2	7.8	7.9	8.15
T_3	*Lactobacillus acidophilus*	7.8	7.7	7.8	7.1	7.5	7.6
T_4	$T_2 + T_3$	7.7	7.6	6.9	6.7	7.1	7.35
T_5	*Lactobacillus bulgaricus + Streptococcus thermophilus*	8.15	8.3	7.6	7.8	7.7	7.9

4. Conclusion

Fermented milk products are considered as the suitable vehicles of probiotics by supplementing them with starter cultures (Parmjit, 2011) [17]. The fermented milk product had the significant amount of vitamin B_{12} along with the probiotic culture of *Propionibacterium freudenreichii*. Hence, this product may serve as a nutraceutical for prevention of vitamin B_{12} deficiency in vegetarians and vegans population, also provides the numerous health benefits of the probiotic cultures upon regular usage. Furthermore, *Propionibacterium freudenreichii* can be used as a starter culture in homemade curd production to improve availability of dietary vitamin B_{12} as the beverage serves higher quantity of vitamin B_{12} than the recommended daily allowance.

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6. Conflict of Interest

Authors declare no conflict of interest.

7. Authors' contribution

HKK conducted the experiments and drafted research paper. SVC provided the guidance and gave critical inputs in correction of manuscript. SB helped in preparation of graphs and tables. RUA helped in review collection and editing the manuscript, BD helped in sensory evaluation process developments, analysis and representation.

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