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Preparation of Vitamin B12-Enriched Curds: A Novel Approach to Address Nutritional Deficiency in Vegetarian Populations

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Abstract

Addressing the significant public health challenge of Vitamin B12 deficiency, particularly within India's substantial vegetarian population (estimated at 35%), this study explored biofortification of the widely consumed dairy product curd (dahi) as a culturally acceptable solution, overcoming barriers of cost, access, and compliance associated with traditional supplements. Researchers isolated bacterial strains from Swiss cheese, presumptively identified as Propionibacterium shermanii based on phenotypic characteristics, and used them to ferment curd under varying conditions, including the presence or absence of supplementary glucose, high or low milk fat content, and two different bacterial isolates. Vitamin B12 concentration was quantified via a colorimetric assay using sodium nitroprusside. The results demonstrated that the maximum yield of Vitamin B12 (7.2 µg/100g) – a 6.5-fold increase over conventional curd (1.1 µg/100g) – was achieved using a specific bacterial isolate (Bacteria 2) in low-fat milk without added glucose. The study found that both the bacterial strain and milk fat content significantly influenced Vitamin B12 production, while supplementary glucose had variable effects depending on the strain used. This proof-of-concept study successfully establishes the feasibility of using propionibacteria to biofortify curd, offering a promising dietary strategy to combat Vitamin B12 deficiency in vegetarian communities. The authors conclude that further research, including molecular identification of the bacterial strains, optimization of production parameters, and human trials to assess bioavailability, is essential to develop this approach into an effective, large-scale nutritional intervention.

Keywords: Vitamin B12, biofortification, presumptive *Propionibacterium shermanii*, fermentation, curd, vegetarian nutrition, functional food

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INTRODUCTION

Vitamin B12 Deficiency: A Global Health Challenge

Cobalamin (vitamin B12) is a micronutrient that is required to yield vital functions in many activities of the body, such as DNA synthesis, red blood cell production, neurobiological activities, and energy metabolism [1]. Vitamin B12 is mostly produced by microorganisms unlike many other vitamins, and it is stored in the animal tissues, thus animal foods are the major sources of Vitamin B12 [2]. This special biosynthetic pathway poses a serious nutritional problem to vegetarian communities around the world, and to which people do not imbibe meat, and in some instances, no animal product whatsoever.

The issue of vitamin B12 deficiency is corroborated as one of the health concerns of the global population that has bothered between 6–20%

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of the general population, and the prevalence rates of the issues exceed those of specific groups [3]. The clinical presentation of the Vitamin B12 deficiency can be either mild or severe and they can entail megaloblastic anemia, fatigue, weakness, constipation, loss of appetite, and weight loss [4]. The neurological symptoms may be either in the form of numbness and tingling in hands and feet, difficulties in balance, depression, confusion, dementia, and memory loss [5]. All the reasons that can lead to the late diagnosis and treatment of Vitamin B12 deficiency and produce an irreparable neurological effect are in the stealthy nature of the condition, its gradual progression, and the absence of specific symptom [6, 7].

Vitamin B12 deficiency has been identified to be high particularly in areas where there are a multitude of vegetarians (based on the latest epidemiological surveys). Among the various vegetarian societies in India, the approximate prevalence of Vitamin B12 deficiency was 47 to 78 percent, it has been reported that the prevalence among vegetarians has been declining despite having a vegetarian diet regardless of their religion, culture, or personal preferences [8, 9]. This worrying number justifies the reality that there is an immediate need to offer effective, culturally acceptable, and sustainable methods to bridge such a nutritional disparity.

The Vegetarian Paradox in India

The situation with Vitamin B12 deficiency in India provides a unique case study in the global environment, given the high number of vegetarians in this country and their specific eating habits. In India, vegetarianism has deep religious and cultural roots, specifically within the adherents of Hinduism, Jainism, and some of the branches of Buddhism [10]. Lacto-vegetarians also include dairy products, which might also supply some Vitamin B12 in its availability; this nutrient in dairy might be inconsistent and not always adequate to satisfy the daily needs [11].

The vegetarian paradox phenomenon in India is an overlapping of the health benefits linked to plant-based diets and the risk of increased predisposition to various nutrient deficiencies, especially Vitamin B12 [12]. Typical Indian vegetarian diets are high in fiber, antioxidants, and phytonutrients and are linked to a lower risk of cardiovascular-related diseases, some cancers, and type 2 diabetes [13]. The same eating habits, however, may result in poor consumption of the nutrients that are mostly found in animal products and thus this gives a nutritional dilemma.

There are several conditions that contribute toward the increased risk of Vitamin B12 deficiency among Indian vegetarians. To begin with, traditional beliefs regarding balanced nutrition have been disintegrated due to fast urbanization and change of diet [13]. Second, the eating pattern of many vegetarians in India has been the same over a series of generations, which can also cause maternal depletion and the intergenerational change of Vitamin B12 status [14]. Third, genetic polymorphisms of Vitamin B12 metabolism seem to be more common in the South Asian population, which may predispose them to deficiency [14].

Not only health but also the economic and societal effects of the widespread Vitamin B12 deficiency in India are beyond personal and individual health. Mothers with Vitamin B12 deficiency have been linked to poor pregnancy because of neural tube defect, intrauterine growth retardation, or developmental delays in newborns [15]. Cognitive development and school achievement in children and adolescents may also be affected by the deficiency [16]. In adult patients, the neurological symptoms of severe deficiency may cause disability and declining productivity of the workforce [17].

Current Approaches to Addressing Vitamin B12 Deficiency

The Vitamin B12 deficiency in the vegetarian population has been dealt with using several strategies, which have their positive and negative aspects. These strategies can be referred to in general as supplementation, fortification, and dietary diversification.

As a Vitamin B12 supplement, it can be direct administration of Vitamin B12 through pills, injections or as a sub-lingual tablet. Although beneficial in managing deficiency, supplementation encounters

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difficulty in terms of access, affordability, adherence and sustainability, especially where the limited resources are insufficient in resource-deprived situations [18]. Vitamin B12 supplements are available and used by only a small number of people in India because of the lack of awareness and low cost, as well as because it lacks systematic supplementation programs and vulnerable populations [19].

Fortification is the addition of Vitamin B12 to food products that are usually consumed throughout the processing process. This has been effectively applied in several nations with breakfast cereals, plant-based milk substitutes, and nutritional yeast common examples of foods to fortify [9]. Food fortification initiatives in India have, however, given special attention to other micronutrients like iron, iodine, and Vitamin A without much attention on Vitamin B12 [8]. Also, availability of commercially fortified products might be restricted in the rural setting and with lower socioeconomic groups.

Vitamin B12 nutrition promotes the intake of food that is rich in Vitamin B12. In the case of vegetarians, it will mostly include more dairy product and as much as will be allowable eggs. Nevertheless, the amount of Vitamin B12 in these foodstuffs can be fluctuating and can be too low to satisfy the needs unless large intakes are taken [6]. Also, the households in India might not access these animal-based food because of the economic constraints.

Due to the shortcomings of the current methodologies, there is increased attention toward considering alternative measures that can be adopted to deal with Vitamin B12 deficiency in vegetarian groups. Biofortification of food foods by microbial fermentation is one of the promising methods that may be used by taking advantage of the capacity of some bacteria to produce Vitamin B12 [20].

Curd: A Traditional Food with Biofortification Potential

Curd, or dahi in India is a fermented product, a milk product which occupies one of the central positions in Indian cuisine and culture. Millions of people worldwide including various regions, social economic classes and ages consume it daily, making it a perfect means of delivering nutrients [21]. The traditional method in preparing curd is the fermentation of milk by lactic acid bacteria mostly the Lactobacillus species in breaking down lactose concurrently to lactic acid, which leads to the trademark tangy flavor and semi-solid texture.

In addition to its cultural importance, curd has also been taken as esteemed due to its nutritional and health advantages. It contains a good amount of protein, calcium, phosphorus and B vitamins and fermentation process increases the bioavailability of those nutrients [22]. Also, curd is known to offer bacteria to gut health through the process of balancing by maintaining the presence of the intestinal microbiota and improving immunity ability [1, 4].

The already established stage of fermentation used in the production of curd presents the possibility of biofortification by introducing bacteria producing Vitamin B12. It is known that certain bacterial species, especially those that are members of the genus Propionibacterium, synthesize Vitamin B12 in the process of fermentation [23]. Using these bacteria in the fermentation of the curd, producing a Vitamin B12 enriched product may be possible, retaining the typical senseless and cultural acceptability of the classic curd.

Propionibacterium shermanii, specifically, has already been reported as a possible source of Vitamin B12 and tends to be present in Swiss cheese, where it is responsible of the typical taste and formation of eyes. The bacteria have a long application history in food production and is widely known as Generally Recognized as Safe (GRAS) by regulators. *P. shermanii* has these characteristics that make it a candidate to be researched to biofortify curd with Vitamin B12.

Research Objectives and Significance

The aim of the given study was to discuss the innovative method of limiting Vitamin B12 deficiency in people who follow the vegetarian diet in India by trying to bioenrich the curd with Vitamin B12 with

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the help of bacteria, which were isolated in Swiss cheese, which were supposedly identified as *Propionibacterium shermanii* due to its phenotypes. The objectives were:

- Isolation of bacteria in Swiss cheese by means of selective medium and identification of bacteria by morphology and biochemical indicators.
- To come up with a biofortified product of curd by using the isolated bacterial strains in fermenting the product.
- To measure and compare Vitamin B12 levels in bio enriched curd samples made with an alternate condition, i.e., variation in milk fat levels, glucose supplementation, and bacteria strain.
- To assess the impact of the following variables on Vitamin B12 production and determine the conditions, which seemed to provide the highest enrichment.

The importance of this study is that it will be able to offer a long-lasting, acceptable, and affordable solution to Vitamin B12 deficiency among vegetarians. This study is beneficial to the larger body of food bio fortification and functional food development by exploiting the historical methods of food and improving these methods through biotechnological methods. The results might guide new attempts to prevent micronutrient deficiencies by food solutions, which is in progress according to the world agenda on nutrition security and health status of the population.

MATERIALS AND METHODS

Experimental Design

In this study, the experimental design applied was a factorial design that sought to examine the influence of three factors on vitamin B12 production in the curd: 1) milk fat content (high-fat and low-fat), 2) glucose breakdown (present and absent), and 3) the bacterial isolate (two different isolates estimated to be *Propionibacterium shermanii*). The above factorial design (2x2x2) would yield 8 different conditions of the experiments as shown in Table 1. All the experimental conditions were run in single, resulting in 8 experimental units.

Table 1. Experimental design showing the eight treatment combinations used in this study.

Sample Code	Glucose Addition	Milk Fat Content	Bacterial Strain
gL1	Present	Low-fat	Bacteria 1
gL2	Present	Low-fat	Bacteria 2
gH1	Present	High-fat	Bacteria 1
gH2	Present	High-fat	Bacteria 2
ngL1	Absent	Low-fat	Bacteria 1
ngL2	Absent	Low-fat	Bacteria 2
ngH1	Absent	High-fat	Bacteria 1
ngH2	Absent	High-fat	Bacteria 2

The primary outcome measure was Vitamin B12 concentration in the fermented curd samples, determined using a colorimetric method.

Materials

Bacterial Strains and Culture Media

- *Swiss Cheese Source:* Swiss cheese (Emmental cheese) was acquired through a commercial retailer. It was the cheese that contained Propionibacterium species specifically, *P. shermanii*, that are known to form the characteristic eyes (holes) of the cheese and that develop the characteristic attribute of flavor of Swiss cheese [24].
- *Culture Media Yeast Extract Lactate (YEL):* YEL agar was selected as the selective media to carry out the isolation and growth of presumptive *Propionibacterium shermanii*. It was prepared in the medium in conventional microbiological procedures.
- *Curd Starter Culture:* The initial acidifiants of milk during curd preparation were a commercial curd starter culture that had lactic acid bacteria.

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Milk Samples

Two types of commercially available pasteurized cow's milk were used in this study.

- Low-fat milk (1.5% fat content).
- High-fat milk (6.0% fat content).

Chemicals and Reagents

The following chemicals and reagents were used in this study:

- Glucose (D-glucose, anhydrous).
- Sodium nitroprusside (for colorimetric assay).
- Gram staining reagents (Crystal violet, Gram's iodine, acetone-alcohol decolorizer, and safranin).
- Phosphate-buffered saline (PBS, pH 7.4).
- Sodium chloride (NaCl).

Methods

Isolation of Bacteria from Swiss Cheese

About 10 g of Swiss cheese was sliced in small and mixed with 90 mL of sterile 0.9% NaCl solution to attain a homogenous suspension. Serial dilutions (10-1 to 10-6) 0.9% NaCl solution was made sterile.

Appropriate dilutions (10-4, 10-5, and 10-6) of aliquots (100 mL) were inoculated on YEL agar plates. The plates were incubated at 30°C at anaerobic condition over a period of 57 days to enable bacteria to have grown.

Following the incubation period, morphologically typical colonies that had morphological features of Propionibacterium (large, round, convex, cream to light brown color) were picked and further investigated. Colony streaking and anaerobic growth of the colonies on fresh YEL agar were 3–5 days at 30°C to get isolated colonies.

Characterization of Isolated Bacterial Strains

The morphological and biochemical characteristics of the isolated colonies were determined using the conventional microbiological techniques.

- *Morphological Characterization:* Morphological characterization was done by Gram staining to identify the Gram reaction, cell morphology, and arrangement. The stained slides were observed using a light microscope.
- *Biochemical Characterization:* The biochemical tests to characterize the isolates were as follows:
  - Catalase test.
  - Nitrate reduction test.
  - Glucose, lactose, sucrose test of fermentation of carbohydrates.

According to these tests of characterization, two different isolates (designated as Bacteria 1 and Bacteria 2) whose characteristics were likely to belong to *Propionibacterium shermanii* were chosen to proceed with experiments. It should be mentioned that no molecular identification procedures, e.g., those grounded on 16S rRNA gene sequencing were carried out, so the identification is merely presumptive relying on the morphological traits.

Preparation of Vitamin B12-Enriched Curd

The preparation of potentially Vitamin B12-enriched curd was carried out using a modified version of the traditional curd-making process, incorporating the isolated bacterial strains. The process involved the following steps.

- *Milk Preparation:* Low-fat (1.5% fat) and high-fat (6.0% fat) milk samples were divided into two groups each: one group supplemented with glucose (1% w/v) and the other without glucose supplementation. The milk was heated to 85°C for 30 minutes for pasteurization and then cooled to 40°C.
- *Inoculation:* Each milk sample (100 mL) was inoculated with.
  - 2% (v/v) commercial curd starter culture.

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  - 1% (v/v) of the isolated bacterial culture (either Bacteria 1 or Bacteria 2, grown in YEL broth for 48 hours).
- *Fermentation:* The inoculated milk samples were incubated at 37°C for the first 6 hours to allow for initial acidification by the lactic acid bacteria, followed by incubation at 30°C for an additional 30 hours. The total fermentation time was 36 hours.
- *Storage:* After fermentation, the curd samples were stored at 4°C until analysis.

Vitamin B12 Extraction and Quantification

The extraction and quantification of Vitamin B12 from curd samples were performed using a colorimetric method based on the reaction with sodium nitroprusside.

Extraction Procedure

- Curd samples (10 g) were homogenized with extraction buffer.
- The homogenate was heated to release protein-bound Vitamin B12.
- After cooling, the mixture was centrifuged.
- The supernatant was filtered and used for Vitamin B12 quantification.

Colorimetric Assay

- Standard solutions of Vitamin B12 were pre-made in the continuum of 0–2.0 mg/mL.
- To 2 mL of all the standard solutions or individual samples extracts, 1 mL of 1% sodium nitroprusside solution was added and stirred, then combined.
- The mixture of reactions was allowed to sit in darkness at room temperature, 15 minutes.
- A spectrophotometer was used to determine the absorbance of 480 nm relative to a reagent blank.
- The standard curve was plotted as a function of the absorbance of the various standard solutions versus the concentration of Vitamin B12.
- The amount of Vitamin B12 in the samples of curd was calculated by interpolating the results of the absorbance on the standard curve and multiplication with the relevant dilution factor (factor of 4).

Recommended Statistical Analysis Approaches

Note: The next statistical methods were not conducted in the original research but can be proposed as the methods that can be used in future research to analyze the data more seriously.

In the case of a factorial design, such as the one adopted in this paper, the statistical methods below would be suitable.

Analysis of Variance (ANOVA)

The main effects and interactions of the experimental variables (milk fat content, glucose supplementation, and bacterial strain) on the requirements of Vitamin B12 could be evaluated with a three-way ANOVA.

This would allow for determination of which factors significantly affect Vitamin B12 concentration and whether there are significant interactions between factors.

- *Post-hoc Tests:* If significant differences are found in the ANOVA, post-hoc tests, such as Tukey's Honestly Significant Difference (HSD) test, could be used for multiple comparisons to identify which specific treatment combinations differ significantly from each other.
- *Correlation Analysis:* Pearson's correlation coefficient could be calculated to assess relationships between different parameters such as between pH and Vitamin B12 concentration.
- *Replication and Error Analysis:* In future studies, performing each experimental condition in triplicate would allow for calculation of means and standard deviations, providing a measure of experimental variability and increasing the reliability of the results.

These statistical approaches would strengthen the analysis and interpretation of the data, allowing for more robust conclusions about the effects of the experimental variables on Vitamin B12 production in curd.

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RESULTS

Isolation and Characterization of Bacterial Strains

Colony Morphology and Microscopic Examination

Two distinct colony types with characteristics consistent with Propionibacterium species were isolated from the Swiss cheese samples on YEL agar plates after anaerobic incubation at 30°C for 5–7 days. The colonies were designated as Bacteria 1 and Bacteria 2 based on their morphological differences.

- *Bacteria 1 (Chalky Colony):* These colonies appeared chalky or dry, cream to light brown in color, circular with entire margins, convex elevation, and approximately 1.5–2.0 mm in diameter after 5 days of incubation.
- *Bacteria 2 (Mucoidal Colony):* These colonies displayed a mucoidal or mucoid appearance, were pale cream in color, circular with entire margins, raised elevation, and approximately 2.0–2.5 mm in diameter after 5 days of incubation.

Microscopic examination of Gram-stained preparations from both colony types revealed Gram-positive, pleomorphic rods with varying morphology. The cells appeared singly, in pairs, or in short chains, consistent with the morphological characteristics typically associated with Propionibacterium species.

Biochemical Characterization

Both isolates exhibited biochemical profiles that were consistent with characteristics typically associated with *Propionibacterium shermanii*, including positive catalase activity, nitrate reduction, and the ability to ferment glucose and lactose.

Based on the morphological and biochemical characteristics, both isolates were presumptively identified as *Propionibacterium shermanii*. It is important to note that molecular identification techniques, such as 16S rRNA gene sequencing, were not performed in this study, so the identification remains presumptive based on phenotypic characteristics only.

Vitamin B12 Content in Curd Samples

Standard Curve for Vitamin B12 Quantification

A standard curve for Vitamin B12 quantification was constructed using standard solutions in the concentration range of 0.1–2.0 µg/mL. The relationship between absorbance at 480 nm and Vitamin B12 concentration showed a linear trend.

Vitamin B12 Concentrations in Different Curd Samples

The Vitamin B12 concentrations in the curd samples prepared under different experimental conditions are presented in Table 2. The values represent the measurements from the colorimetric assay, with the final concentrations calculated after applying the dilution factor of 4.

The highest Vitamin B12 concentration (7.2 µg/100 g) was observed in the sample prepared with low-fat milk, no glucose addition, and Bacteria 2 (ngL2). This concentration was higher than all other treatment combinations and represented a 6.5-fold increase compared to the control curd (1.1 µg/100 g) prepared without the addition of the isolated bacterial strains.

Table 2. Vitamin B12 concentration in curd samples prepared under different experimental conditions.

Sample Code	Glucose Addition	Milk Fat Content	Bacterial Strain	Optical Density at 480 nm	Vitamin B12 Concentration (µg/100 g)*
ngL2	Absent	Low-fat	Bacteria 2	0.073	7.2
gL1	Present	Low-fat	Bacteria 1	0.055	5.6
gH1	Present	High-fat	Bacteria 1	0.048	4.8
ngL1	Absent	Low-fat	Bacteria 1	0.047	4.8
gH2	Present	High-fat	Bacteria 2	0.042	4.2

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gL2	Present	Low-fat	Bacteria 2	0.037	3.8
ngH2	Absent	High-fat	Bacteria 2	0.019	1.8
ngH1	Absent	High-fat	Bacteria 1	0.018	1.6
Control	–	Low-fat	None	0.011	1.1

Note: *The Vitamin B12 concentrations were calculated using the standard curve equation and multiplied by the dilution factor of 4.

The second highest Vitamin B12 concentration (5.6 μg/100 g) was found in the sample prepared with low-fat milk, glucose addition, and Bacteria 1 (gL1), followed by samples gH1 and ngL1 (both 4.8 μg/100 g). The lowest Vitamin B12 concentrations were observed in samples prepared with high-fat milk and no glucose addition (ngH1 and ngH2), with values of 1.6 and 1.8 μg/100 g, respectively.

Observed Patterns in Vitamin B12 Production

Based on the raw data, several patterns were observed regarding the influence of the experimental variables on Vitamin B12 concentration.

- *Milk Fat Content:* In general, samples prepared with low-fat milk showed higher Vitamin B12 concentrations compared to those prepared with high-fat milk, when other variables were held constant. For example:
  - ngL2 (7.2 μg/100 g) vs. ngH2 (1.8 μg/100 g).
  - gL1 (5.6 μg/100 g) vs. gH1 (4.8 μg/100 g).
  - ngL1 (4.8 μg/100 g) vs. ngH1 (1.6 μg/100 g).
  - gL2 (3.8 μg/100 g) vs. gH2 (4.2 μg/100 g) [Note: This pair is an exception to the general pattern]
- *Glucose Addition:* The effect of glucose addition appeared to vary depending on the bacterial strain used.
- *For Bacteria 1:* Glucose addition generally resulted in higher Vitamin B12 concentrations in both low-fat and high-fat milk conditions.
  - gL1 (5.6 μg/100 g) vs. ngL1 (4.8 μg/100 g).
  - gH1 (4.8 μg/100 g) vs. ngH1 (1.6 μg/100 g).
- *For Bacteria 2:* Glucose addition resulted in lower Vitamin B12 concentrations in low-fat milk but higher concentrations in high-fat milk.
  - gL2 (3.8 μg/100 g) vs. ngL2 (7.2 μg/100 g).
  - gH2 (4.2 μg/100 g) vs. ngH2 (1.8 μg/100 g).
- *Bacterial Strain:* The relative performance of the two bacterial strains in terms of Vitamin B12 production varied across different conditions.
- *In low-fat milk without glucose:* Bacteria 2 produced more Vitamin B12 than Bacteria 1.
  - ngL2 (7.2 μg/100g) vs. ngL1 (4.8 μg/100 g).
- *In low-fat milk with glucose:* Bacteria 1 produced more Vitamin B12 than Bacteria 2.
  - gL1 (5.6 μg/100g) vs. gL2 (3.8 μg/100 g).
- *In high-fat milk with glucose:* Bacteria 1 produced more Vitamin B12 than Bacteria 2.
  - gH1 (4.8 μg/100g) vs. gH2 (4.2 μg/100 g).
- *In high-fat milk without glucose:* Bacteria 2 produced slightly more Vitamin B12 than Bacteria 1.
  - ngH2 (1.8 μg/100g) vs. ngH1 (1.6 μg/100 g).

These observations suggest complex interactions between the experimental variables, with milk fat content, glucose addition, and bacterial strain all appearing to influence Vitamin B12 production in the curd samples.

Recommended Data Analysis Approaches

Note: The following analyses were not performed in the original study but are suggested as approaches that could be applied to analyze the data more rigorously in future work.

Visualization of Results

To better understand and communicate the patterns in the data, the following visualizations could be created.

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- *Bar Graph of Vitamin B12 Concentrations:* A bar graph showing the Vitamin B12 concentration for each experimental condition would provide a clear visual comparison of the results. The bars could be grouped by milk fat content and glucose addition, with different colors representing the two bacterial strains.
- *Interaction Plot:* An interaction plot showing how the effect of one variable (e.g., glucose addition) on Vitamin B12 concentration depends on the level of another variable (e.g., bacterial strain) would help visualize the complex interactions observed in the data.
- *Main Effects Plot:* A main effects plot showing the average effect of each variable (milk fat content, glucose addition, bacterial strain) on Vitamin B12 concentration would help identify the overall influence of each factor.

Statistical Analysis

To determine the statistical significance of the observed patterns, the following analyses could be performed.

- *Three-way ANOVA:* A three-way ANOVA would test for significant main effects and interactions of milk fat content, glucose addition, and bacterial strain on Vitamin B12 concentration. This would help determine which factors and interactions significantly influence Vitamin B12 production.
- *Post-hoc Tests:* If significant effects are found in the ANOVA, post-hoc tests, such as Tukey's HSD test, could be used to identify specific differences between treatment combinations.
- *Effect Size Calculation:* Calculating effect sizes (e.g., partial eta-squared) would provide information about the magnitude of each factor's influence on Vitamin B12 production, complementing the significance testing.

These analyses would provide a more rigorous evaluation of the patterns observed in the data and strengthen the conclusions drawn from the study.

DISCUSSION

Isolation and Presumptive Identification of Bacterial Strains

In this research, two different bacterial isolates of Swiss cheese with the morphological and biochemical properties that matched to *Propionibacterium shermanii* were isolated. This isolation method with YEL agar serving as a selective medium enabled the growth of bacteria with similar phenotypic characteristics as literature reports on Propionibacterium species [25].

The two isolates which were termed as Bacteria 1 and Bacteria 2 had different colony morphologies (chalky and mucoidal) and had similar biochemical profiles. This phenotypic variation is in line with the diversity that has been described in Propionibacterium species [26]. Nevertheless, the identification in this work was presumptive in nature, in which it was made relying only on the phenotypic features. The study did not use the methods of molecular identification, like 16S rRNA gene sequencing, that would give conclusive evidence on the identity of the species.

Restrictions of the phenotypic identification procedures have long been known in microbiology. Although it can be useful, traditional biochemical and morphological tests do not necessarily qualify as reliable in identifying between closely related species or strains. This specifically applies to the genus Propionibacterium which has been re-taxonomically revised over the past several years [27, 28]. Hence, the strains of bacterial species utilized in the present study can be termed as presumptively identified in waiting of a molecular confirmation.

Vitamin B12 Production in Biofortified Curd

Influence of Milk Fat Content

We have found that this was the trend in our finding with low-fat milk tending to provide much higher levels of Vitamin B12 than in high-fat milk and we considered these various experimental circumstances. The level of this was observed especially when comparing ngL2 (7.2 mg/100 g) and ngH2 (1.8 mg/100 g) samples, which did not differ in milk fat content, though the concentration of Vitamin B12 varied four times.

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A hint of the inverse correlative relation between milk fat content and Vitamin B12 production that was found in this study is in line with existing studies on milk handling and the metabolic activity of Propionibacterium in the dairy habitats [29]. Stated cheese models with high fat content prevented the growth of *P. freudenreichii*, possibly because of a decreased availability of nutrients soluble in water or a different rate of oxygen availability. On the same note [30], found that lipid oxidation products in high-fat dairy systems may suppress the metabolic activity of propionibacterium.

Essentially, several of these cofactors and precursors, such as aminolevulinic acid, cobalt ions, and dimethyl benzimidazole, are necessary in synthesizing Vitamin B12, in a biochemical perspective [8]. These water-soluble components may be less available and less accessible in a high-fat environment because of the effect of partitioning or the creation of fat globule membranes by which the nutrient diffusion is blocked [31].

Effect of Glucose Supplementation

The influence of the glucose supplementation on the formation of Vitamin B12 was rather multifaceted and it seemed to depend on the bacterial strain under which the study was conducted. In Bacteria 1, glucose supplementing generally led to the increase of Vitamin B12 concentrations as seen in shows of higher values in gL1 (5.6 mg/100 g) than in ngL1 (4.8 mg/100 g). Bacteria 2 on the other hand indicated more Vitamin B12 in the low-fat milk lactose-free condition with glucose, and ngL2 (7.2 mg/100 g) results achieving significantly greater levels as compared to gL2 (3.8 mg/100 g).

This glucose supplementation response is strain-dependent, a phenomenon that indicates variation in the level of carbon metabolism of the two isolates. The above studies have also indicated that Vitamin B12 production in Propionibacterium is either promoted or inhibited by glucose based on the strain of the microbe and the conditions of the culture [32, 33].

Enhanced metabolic activity and an increase in biomass production can be given as the possible reason behind the stimulatory impact of glucose on Vitamin B12 production in Bacteria 1. The glucose is a readily and readily accessible source of carbon which can be quickly processed through the glycolytic pathway generating energy and cell growth and biosynthetic precursors [7].

In contrast, the apparent neutralizing influence of glucose on Vitamin B12 production by Bacteria 2 in low-fat milk condition could be described by the effect of catabolite reolder. Hyperextensive amounts of glucose can suppress genes that participate in the utilization of the alternative carbon sources and the production of the secondary metabolites such as the production of Vitamin B12 [28].

Strain-Specific Differences in Vitamin B12 Production

We observed that there were evident variations in Vitamin B12 production in under the two isolated bacterial strains with Bacteria 2 having a better production effectiveness in under certain circumstances (low-fat milk, absent glucose). This strain specific difference in Vitamin B12 production agrees with the prior research that have indicated that there is significant variation in Vitamin B12 yield in various strains of Propionibacterium [25].

The identified variation can be explained by genetic peculiarities in the biosynthetic route of Vitamin B12 comprising of about 30 genes and it is among the most complicated pathways of the synthesis of vitamin in bacteria [31]. The expression and regulation of these genes and the differences in the activity of major enzymes that participate in the pathway may contribute to the difference in the Vitamin B12 production in this study.

There can also be a difference in adaptation of the two strains in the curd environment such as acid tolerance, lactose and other milk utilization as well as the interaction of the strains with the lactic acid bacteria within the starter culture. These influences may have an indirect effect on Vitamin B12 synthesis by intervening with the growth and metabolic activity of bacterial strains of the fermented product.

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Comparison with Traditional Curd

The vitamin B12 maximum in this present study (7.2 mg/100 g in the ngL2 sample) was fool seven point five times more than that of the untreated control curd (1.1 mg/100 g) that had not been brought to Vitamin B12 using the isolates. This improvement shows the possibility of the biofortification methodology used in this paper [34].

To contextualize the results in relation to Vitamin B12, the recommended dietary allowance (RDA) of 2.4 mg daily is the recommended amount of Vitamin B12 per day (Institute of Medicine, 1998). The biodrained curd produced in this study would contain about 300 per cent of the RDA in 100 g serving size, and the curd would end up being a likely good food source of this vital nutrient within the diet. Comparatively, traditional curd would just offer approximately 46 percent of RDA on 100 g of one serving.

The biofortified curd produced in this study will be superior when compared to most of the animal-based foods when compared to other natural sources of Vitamin B12. As an example, our biofortified curd (7.2 mg /100 g) has a higher amount of Vitamin B12 than milk (0.4 mg/100 g), yogurt (0.2 mg/100 g), and eggs (1.1 mg/100 g) and is like some fish species (2–10 mg/100 g) [35].

Limitations of the Study

Although this experiment proved the possibility of biofortifying curd with Vitamin B12 with the help of bacterial strains obtained by the isolation of bacterial strains on Swiss cheese, some significant limitations must be noted.

- *Presumptive Bacterial Identification:* The bacterial isolates that were employed in this study were merely identified based on phenotypic characteristics. Molecular identification tests that would have given conclusive results on the identity of the species including 16S rRNA gene sequencing, were not carried out.
- *Single Measurements,* one measurement was made of each of the experimental conditions, without repetitions. This hinders the distribution of variability and reproducibility of the results, and it prevents statistical analysis of the data.
- *Limited Analytical Validation:* The colorimetric technique used in Vitamin B12 quantification was not fully validated in comparison with reference techniques because of microbiological assay, HPLC, or LC-MS. This leads to the concerns of specificity and accuracy in the measurements of Vitamin B12.
- *No Bioavailability Assessment:* The bioavailability of the Vitamin B12 generated in the biofortified curd was not analyzed. Vitamin B12 being present in the curd does not in any way guarantee the effective absorption and usage of the compound by the human body.
- *No Sensory Assessment:* The organoleptic characteristics of the bio fortified curd were not evaluated. Acceptance of any biofortified food product by the consumers is significant towards its practical use.
- *No Stability Studies:* The vitamin B12 stability in the biofortified curd as the storage time was not studied. Vitamin B12 can be susceptible to a range of environmental factors and retention of this vitamin during storage is also one of the factors to consider in real life applications.

Such limitations point out the stage of the present research and indicate the necessity of the further research to prove and develop these findings.

Recommended Future Research Directions

In terms of the research findings and limitations, it can be suggested the following research directions in the future.

- *Molecular Identification of Bacterial Strains: Future Research:* The future research ought to be utilized molecular methods, like 16S rRNA gene sequencing, as a way of proving the identity of the bacteria strains used in biofortification. This would give a more assurance as to which organisms produced Vitamin B12 and would be able to easily compare other studies.
- *Experimental Replication and Statistical Analysis:* Repetition of the experiments with several replicates would mean that the data can be statistically analyzed to give more reliable evidence on the effects of the experimental variables on Vitamin B12 production.

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- *Advanced Analytical Methods:* It would be better to establish whether the colorimetric method is validated by reference methods, like HPLC or LC-MS, to get a better confidence in the measurement of Vitamin B12. These methods might also be able to differentiate between various types of Vitamins B12, the possible analogues, or precursors.
- *Bioavailability Studies:* In vitro digestion model or human intervention Studies is to determine bioavailability of the Vitamin B12 in biofortified curd would be useful in determining nutritional effects of the product.
- *Sensory Assessment and Consumer Acceptance:* It will involve formal sensory assessment of the bio fortified curd and consumer acceptance of the product that will give an insight of the practical potential of the product in terms of addressing Vitamin B12 deficiency.
- *Stability Studies:* The study of Vitamin B12 stability of the biofortified curd under various conditions would guide the handling, packaging as well as distribution.
- *Process Optimization:* It would be possible to further improve the production of Vitamin B12 through systematic optimization of the biofortification process by exploring more variables, including fermentation time, temperature profiles, and starter culture composition.
- *Scale-up Studies:* The research into the viability of scaling-up the process of biofortification into pilot or commercial scale would resolve the issue regarding large scale production in practice.

These research directions would be a continuation of the initial findings of this paper and the formation of a practical effective solution to the Vitamin B12 deficiency problem using biofortified curd.

CONCLUSIONS

Summary of Key Findings

This preliminary paper examined the possibility of biofortifying curd with Vitamin B12 by using bacterial isolates obtained through detection of Swiss cheese and assumed to be *Propionibacterium shermanii* based on morphological features. The study examined the influence of milk fat level, glucose supplementation and bacterial strain on the Vitamin B12 synthesis in curd.

The key findings of this study can be summarized as follows.

- *Propionibacterium shermanii* 2 discrete bacterial cultures were isolated and presumptively identified based on morphological and biochemical properties as Swiss cheese. The isolates would have varied morphologies of colony (chalky and mucoidal), and yet their biochemical profiles were similar.
- Optimal levels of Vitamin B12 were obtained in curd that was prepared using low-fat milk, and in the absence of glucose additions, as well as in the bacterial growth characterized by the name of the bacteria 2. It is a 6.5-fold increase over traditional curd (1.1 mg/100 g) and would contain about 300 percent of the suggested daily recommendable levels of Vitamin B12 in 100 g portion.
- Low-fat milk tended to have a higher concentration of Vitamin B12 than the high-fat milk so there was an inverse correlation of the concentration of milk fat and Vitamin B12 produced by the bacteria strains.
- The growth-dependent effect of glucose supplement on vitamin B12 production was found to be dependent on the strain with increased production by bacteria Bacteria 1 and inconsistent effects on bacteria Bacteria 2 with milk fat content.
- The patterns of Vitamin B12 production observed in the two bacterial strains varied by each of the various experimental conditions with Bacteria 2 exhibiting better Vitamin B12 production at certain experimental conditions (low-fat milk, no glucose).

These results indicate that it is possible to fortify curd with Vitamin B12 by bacteria fermentation and this approach may potentially be the source of the substantial amount of this vital nutrient in the diet. Nonetheless, the consideration of this study and its drawbacks is that it is preliminary.

Implications for Addressing Vitamin B12 Deficiency

Vitamin B12 fortified curd has the potential implication of improving the ill level of Vitamin B12 deficiency among the vegetarian communities, especially in India and other countries with similar diets. By

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fortifying a popular and culturally accepted foodstuff with Vitamin B12, this solution may be beneficial in several ways as compared to the more traditional modes of solving micronutrient deficiencies.

This contrasts with supplementation, which is sometimes accompanied by issues of access, affordability, and association, since bio fortified curd may be incorporated into the existing dietary habits without necessarily becoming a behavioral intervention. The biofortification application of the technique uses traditional food preparation and locally accessible food materials and may be a more viable method of fortification compared to industrial fortification with synthetic vitamins, and the process may also be culturally acceptable.

Nutritionally, the biofortified curd that is developed in this paper has potentials of making a substantial contribution to the daily Vitamin B12 needs. It should be mentioned though that the bioavailability of the Vitamin B12 in the biofortified curd was not determined in this study and this issue would require additional investigation in the future to determine its nutritional effects.

Recommended Future Research Directions

Although such research gives preliminary evidence of the possibility of biofortifying curd with Vitamin B12, there are a few points, which should be developed more thoroughly to proceed with such practice and achieve the most significant effect. The suggested future studies are:

- *Molecular Identification of Bacterial Strains:* The use of molecular applications, like 16S rRNA gene sequence, to determine categorically the bacterial strains that will be employed in biofortification.
- *Experimental Replication and Statistical Analysis:* To be able to do statistical analysis of the data and should have a stronger conclusion, the experiments should be reproduced in several replicates.
- *Techniques of Further Analysis:* To develop more confidence in the Vitamin B12 measurements, one of the possible solutions could be to validate the colorimetric technique against reference techniques (HPLC or LC-MS).
- *Bioavailability Studies:* Researching into the bioavailability of the Vitamin B12 in biofortified curd by in vitro models of digestion or studying bioavailability using human beings.
- *Sensory Evaluation and Consumer Acceptance:* This will involve what is termed as formal sensory evaluation of the biofortified curd and the consumer acceptance.
- *Stability Studies:* The stability of Vitamin B12 in the bio-enriched curd stored at various conditions.
- *Process Optimization:* Scientific optimization in the bio fortification process, through understanding other variables, like fermentation time, temperature profiles, and starter culture make-up.
- *Scale-up Studies:* Exploring scaling up of the biofortification process: From laboratory to pilot or commercial scale.

Concluding Remarks

The initial research shows that biofortification of curd with Vitamin B12 using bacterial fermentation is a viable method to solve the Vitamin B12 deficiency in vegetarian groups. The high percentage of Vitamin B12 content that was obtained in this study will indicate that the methodology could be used to improve the nutritional state of at-risk groups.

Nevertheless, the weaknesses of this research such as the presumptive designation of the bacterial strains, no experimental replicability, minimal analytical validation as well as the non-conduction of bioavailability testing point to the fact that more research would be necessary before this study can be turned into practice.

Once more research concerning these limitations is done, and the recommended areas of future effort have been elaborated on, biofortified curd may have a very useful role to play in world in the fight against micronutrient deficiencies and jeopardy toward fuller nutrition of the entire world.

Blending traditional food practices with biotechnology methods as in this case is an encouraging policy of coming up with culturally oriented solutions to modernity nutritional problems.

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