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Cholesterol Reduction and Vitamin B12 Production Study on Enterococcus faecium and Lactobacillus pentosus Isolated from Yoghurt

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Abstract: The present study was aimed to test cholesterol reduction and vitamin B12 production abilities of the isolated lactic acid bacteria (LAB). Three LAB isolates, namely, Enterococcus faecium (EF), Enterococcus faecium (Chole1), and Lactobacillus pentosus (7MP), having probiotic potential, were isolated from yoghurt. These isolates were screened for bile salt hydrolase (BSH) activity, cholesterol reduction property in MRS broth, and the production of vitamin B12. The present study revealed that the isolate 7MP possesses the highest potential of (48%) cholesterol reduction compared to the other isolates. The isolates EF and Chole1 produced a good amount of (1 ng/mL) vitamin B12. These isolates were identified by 16S rRNA gene sequencing and confirmed by MALD_TOF analysis. Thus, the use of these LAB isolates for yoghurt-making can offer the value addition of lowering cholesterol and vitamin B12 fortification in fermented food.

Keywords: cholesterol reduction; Enterococcus faecium; Lactobacillus pentosus; vitamin B12 production; yoghurt

1. Introduction

Humans have consumed fermented foods laden with beneficial bacteria with probiotic properties for thousands of years. Thus, humans have unknowingly consumed these beneficial bacteria with traditionally fermented foods since ancient times. Lactic acid bacteria (LAB) were used to ferment food from those ages [1]. These bacteria have been studied and are clinically effective in treating many disease conditions [2]. Fermented foods, thus being the foremost consumed processed food by humans, are also valued due to their enhanced shelf life, safety, nutrition, and other properties [3]. As many fermented foods are loaded with live microorganisms with probiotic properties, they add functionality to the food. The fermenting microorganisms modify the essential ingredients of food and establish new bioactive compounds. The microorganisms associated with fermented dairy, vegetable products, and cereals foods are LAB from the genera Lactobacillus, Pediococcus, Enterococcus, Leuconostoc, etc. [4].
In recent years, foods have been consumed to ensure appetite and nutrition and improve the consumer’s health. This usage, described with the term “functional food,” involves food with components that offer additional health benefits such as probiotic effects and cholesterol reduction properties [1].

Since an increase in blood/serum cholesterol is a significant factor responsible for coronary heart disease [5], LAB are gaining significance as cholesterol reducers [6]. Elevated serum cholesterol levels are treated by hypocholesterolemic drug therapy, dietary interventions, etc. However, the chemotherapeutics approach may pose side effects.

Vitamin B\textsubscript{12}, a water-soluble vitamin, is an essential molecule for human nutrition and its dietary reference intake (D.R.I.) of 2.4/day in adults is a general selected value [7]. Vitamin B\textsubscript{12} deficiency is a general problem worldwide, leading to many clinical conditions. Animals, plants, and fungi cannot synthesize this vitamin; it is produced by microorganisms [1]. LAB are known to produce a wide range of this water-soluble vitamin.

LAB are reported to offer many functional properties to the food that they ferment. However, the beneficial effect is species-/strain-specific. Hence, the present study aimed to assess the cholesterol reduction and vitamin production of isolates \textit{Enterococcus faecium} and \textit{Lactobacillus pentosus} from traditionally fermented food, yoghurt, and their identification based on 16S rRNA and confirmation by MALDI-TOF MS.

2. Materials and Methods

2.1. Bacterial Isolates, Culture Media, and Growth Conditions

Various types of fermented yoghurt samples were processed for isolation of LAB. Three LAB isolates, namely, (i) \textit{E. faecium} (EF), (ii) \textit{E. faecium} (Chole1), and (iii) \textit{L. pentosus} (7MP), were obtained from different yoghurt samples [8]. The cultures were grown on an MRS medium (Hi-Media, Mumbai, India) and incubated under microaerobic conditions (created by burning a candle; the candle’s flame burns until extinguished by oxygen deprivation, creating a carbon dioxide-rich, oxygen-poor atmosphere) in a sealed desiccator at 37 °C for 24 to 48 h. Vitamin B\textsubscript{12} auxotroph mutant culture of \textit{E. coli} (Davis A mutant strain 113-3D) was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Vitamin B\textsubscript{12} (trade name = Optineuron) was obtained from LUPIN Ltd., Tarapur, India.

2.2. Cholesterol Reduction Activity

2.2.1. Bile Salt Hydrolase (BSH) Activity Testing

The BSH activity of isolates was checked according to the method of Kunzes and Bhalla [9], with a slight change. The fresh growth of isolates from MRS broth (10 µL) with 10\textsuperscript{10} cfu/mL was inoculated on BSH test medium (MRS medium supplemented with CaCl\textsubscript{2} and bile salt). The plates were incubated at 37 °C for 48 h incubation, and the diameter of halos/opaque precipitation around colonies was taken as an indication of BSH activity.

2.2.2. Cholesterol Reduction

The assessment of cholesterol reduction of the isolates was carried out by separately growing the cultures in MRS broth supplemented with cholesterol. The assay was based on the amount of cholesterol reduced by the isolate. The amount of cholesterol reduced was determined by the enzymatic method [10]. In short, into the MRS medium (1 mL) containing 200 µg/mL cholesterol (filter sterilized) from the kit (AUTOSPAN), a 50 µL of fresh log phase culture (10\textsuperscript{10} cfu/mL) isolate was inoculated. Sterile distilled water was added to the tube labeled as control. After 24 h incubation at 37 °C, cells were separated by centrifugation at 5000 \times g for 10 min at 8 °C. The cell mass and supernatant were separated, and the cholesterol in the supernatant was estimated following the AUTOSPAN enzymatic method [10]. The amount of cholesterol content reduced in the test was determined using the following formula and expressed in µg/mL:

\[
\text{Cholesterol reduced (µg/mL)} = \text{Cholesterol in Control} - \text{Cholesterol in the test.}
\]
2.3. Vitamin B₁₂ Production Assay

For this purpose, the log phase culture of each isolate was separately grown on vitamin B₁₂ bioassay medium (Hi-Media Mumbai, India) at 37 °C for 24–48 h and observed for the appearance of growth. The ability of an organism to grow in the absence of vitamin B₁₂ was considered its capacity to synthesize vitamin B₁₂ [11].

2.3.1. Cells Extract Method

Having confirmed the ability of an organism to produce vitamin B₁₂, the cultures were subjected to cell lysis and extraction of vitamin B₁₂ followed by an estimation of vitamin B₁₂. For this purpose, the cell mass of each isolate from the 48 h incubated broth was separated by centrifugation 5000 × g for 10 min at 8 °C, and the cells were suspended in ice-chilled sterile distilled water, mixed vigorously, and re-centrifuged at 5000 × g for 10 min at 8 °C. The cell pellet was separated, and 180 µL of sterile distilled water was added to make the slurry. This slurry was thoroughly mixed with 20 µL sterile 0.01% Tween 80 (Merck, India) and incubated at −20 °C for 2 h followed by thawing with shaking at 37 °C. This mixture was subjected to freezing at −20 °C for 15 h and then re-thawed to get dead cell extract slurry. This slurry was used as a sample for the vitamin B₁₂ estimation.

2.3.2. Vitamin B₁₂ Bioassay

A vitamin B₁₂ bioassay was performed according to Madhu et al. [12] and Santos et al. [13], with some modifications. For the diffusion assay, vitamin B₁₂ auxotroph mutant (Davis A mutant strain 113-3D, 0.5 OD at 615 nm) was poured into a vitamin bioassay medium. Plates were allowed to solidify, and 8 mm wells were bored (labeled as test, standard, and control). In each well in the test set, 25 µL of the slurry sample was added; in the standard set 25 µL of varying concentrations of the standard vitamin B₁₂ sample (1–10 ng/mL) was added; and in the control set, sterile distilled water (25 µL) was added. The assay was performed in triplicate. All three sets were kept in a freezer for pre-diffusion and then incubated at 37 °C for 24 h. Following the incubation, the zone diameter of the growth was measured. From the standard graph straight line equation, vitamin B₁₂ content in the sample was estimated as vitamin B₁₂ produced per mL [12,13].

2.3.3. Influence of Glycerol on Vitamin B₁₂ Production

To check the effect of glycerol on vitamin B₁₂ production, a 0.5% (v/v) glycerol was added to the vitamin B₁₂ growth medium. Cultures were grown in glycerol-amended medium at 37 °C for 24 h, followed by cell extraction and vitamin B₁₂ bioassay [13].

2.4. Identification of Isolates

2.4.1. Molecular Identification

Molecular identification of LAB isolates was carried out earlier by using the 16S rRNA sequencing method. The sequencing of the 16S rRNA genes of the isolate was carried out according to the method of Gangurde et al. [14]. Genomic DNA of the isolates was extracted according to the method of Sambrook [15] by using a DNA Miniprep Purification Spin Kit (Hi-Media, Mumbai, India). The 16S rRNA genomic region was amplified with the help of universal primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′) in a Gene Amplifier PCR System 9700 (Perkin Elmer, USA) [16]. The amplified sequences were analyzed with gapped BLASTn and compared with the NCBI database [17] and EzTaxon [18]. The evolutionary history was inferred using the neighbor-joining method [19]. The evolutionary distances were computed using the Kimura 2-parameter method [20]. All ambiguous positions were deleted for each sequence pair. Evolutionary analyses were conducted in MEGA X [21].

2.4.2. MALDI-TOF-MS Identification

Only two isolates, namely, EF and Chole1, exhibited B₁₂ production; hence, they were subjected to identification confirmation by the MALDI-TOF-MS method. These cultures
were subjected to confirmatory identification by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. MALDI-TOF-MS identification was carried out according to the ethanol-formic acid extraction method [22]. The spectra analysis of this study was indicated by BioTyper log (score), and according to the scores, species-level identification was confirmed [23]. A small amount (0.1 mL) of pure culture of the isolate was picked using a sterile toothpick and applied to the MALDI target plate. Then, 1 μL saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution was added to it. The matrix was prepared using a matrix solvent that consisted of acetonitrile, trifluoroacetic acid, and Mill-Q water. From this solvent, 250 μL was added to the vial containing 200 μg HCCA and vortexed for 10 min until the solution became clear. The matrix was allowed to dry at 28 °C; the plate was inserted into the instrument. The spectra were calibrated externally using the standard calibrant mixture. The MALDI biotype software 3.0 (Bruker Daltonik, Leipzig, Germany) was used for the MALDI-TOF identification. A short extraction protocol was followed for the samples with non-reliable results and nospectra. The identification results are expressed by the BiTyper log (score).

2.5. Statistical Analysis

All the experiments were performed in triplicates, and the average of three replicates was analyzed by one-way analysis of variance followed by Tukey’s HSD test [24].

3. Results

3.1. Cholesterol Reduction Activity

The growth of isolates on the BSH test medium resulted in the formation of halos/an opaque precipitation zone formed around the growth of all the tested cultures (Figure 1). The isolate 7MP produced a larger precipitation zone, and thus, it was considered to have maximum BSH activity compared to the other isolates. It was also found that all three isolates solubilized/reduced cholesterol in the broth medium (Figure 2). The level of cholesterol reduction by all three isolates ranged between 42 to 48%. The isolate 7MP exhibited a higher cholesterol reduction activity of 47.32% compared to the other two isolates. This higher activity of cholesterol reduction is a good feature as far as the beneficial effect is concerned.

![Figure 1. BSH activity of isolates.](image-url)
NCBI GenBank. Thus, LAB cultures were identified as *Lactobacillus pentosus* with the accession number KX886791 and KX886789 [8].

### 3.2. Vitamin B₁₂ Production

Good growth of the isolates *E. faecium* (EF) and *E. faecium* (Chole1) was observed on a vitamin B₁₂-free medium. This growth on the B₁₂-free medium indicated the vitamin B₁₂-producing abilities of the isolates *E. faecium* (EF) and *E. faecium* (Chole1). Both the isolates EF and Chole1 produced 1 ng/mL vitamin B₁₂ (Figure 3), whereas the isolate *L. pentosus* (7MP) did not grow on the B₁₂-free medium. This indicated its inability to produce the vitamin B₁₂ required for its growth, and hence it was considered a non-producer of vitamin B₁₂.

**Figure 3.** Vitamin B₁₂ production by LAB isolates.

**Supplementation of glycerol in the growth medium resulted in a slight improvement in vitamin B₁₂ production. This supplementation improved vitamin B₁₂ by 21% and 15% in EF and Chole1, respectively (Figure 3).**

### 3.3. Influence of Glycerol on Vitamin B₁₂ Production

Supplementation of glycerol in the growth medium resulted in a slight improvement in vitamin B₁₂ production. This supplementation improved vitamin B₁₂ by 21% and 15% in EF and Chole1, respectively (Figure 3).

### 3.4. Identification of Isolates

#### 3.4.1. Molecular Identification

The comparison of 16S rRNA gene sequences of isolates Chole1 and EF using an NCBI BLAST search revealed 99.93% and 100% similarity, respectively, with the sequence of *Enterococcus faecium* CGMCC 1.2136 (T) (Figure 4), and 7MP revealed 99.33% similarity with *Lactobacillus pentosus* JCM 1558 (T) (Figure 5) with the sequences available in the NCBI BLAST search.
GenBank. Thus, LAB cultures were identified as *Enterococcus faecium* and *Lactobacillus pentosus*, and their 16SrRNA gene sequences were earlier deposited in NCBI GenBank under the accession number KX886791 and KX886789 [8].

![Phylogenetic tree of isolate Chole1](image1)

**Figure 4.** Phylogenetic tree of isolate Chole1.

![Phylogenetic tree of isolate 7MP](image2)

**Figure 5.** Phylogenetic tree of isolate 7MP.
The evolutionary history was inferred using the neighbor-joining method. The evolutionary distances were computed using the Kimura two-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1443 positions in the final dataset. Evolutionary analyses were conducted in MEGA X software.

3.4.2. MALDI-TOF-MS Study of Bacterial Isolates from Yoghurt

Though 16S rRNA gene sequencing is the accepted standard method in bacterial taxonomy, an additional proteomics-based study on protein profiling with MALDI-TOF-MS has been used as a means of rapid identification of Enterococcus spp. with high throughput and sensitivity [19]. The MALDI-TOF mass spectra measurement of isolate samples with Biotyper is shown in Figure 6A,B. The isolate presenting a $\geq 1.7$ log value with isolate in the database was identified as the affiliate of that genus and isolates revealing $\geq 2.0$ log values were identified as belonging to that species. The EF and Chole1 were 2.204 and 2.322, respectively; thus, it confirms the isolates EF and Chole1 as Enterococcus faecium. The results of MALDI-TOF MS matched very well with the results of the 16S rRNA gene sequence analysis.

![Figure 6](image_url)

**Figure 6.** MALDI-TOF-MS spectra of isolate (A) EF and (B) Chole1.
4. Discussion

Although most vitamins are present in various foods consumed by humans, malnutrition, vitamin deficiency, or insufficient absorption/intake of vitamins are still the cause of vitamin deficiencies in humans in most developing countries [23]. Vitamin B\textsubscript{12} is a complex compound that is naturally produced by specific LAB. Thus, the enrichment of foods, particularly dairy foods, with vitamin B\textsubscript{12} appears to be one of the best approaches to providing a good source of vitamin B\textsubscript{12} [23].

Hypercholesterolemia is one of the major causes of cardiovascular disease. Supplementation with probiotic bacteria contributing to improved lipid metabolism is one of the best measures to combat hypercholesterolemia [23].

Cholesterol metabolism is controlled by the actions of \textit{Lactobacillus} species on microflora and the general activity of human gut microbiota [25]. This activity involves the deconjugation of bile salt, the concentration of short-chain fatty acids, and the ratio of propionate. These activities help provoke the cholesterol-lowering effect of LAB cultures such as \textit{Lactobacillus}, that are reported to exhibit extensive BSH activity. Hence, those cultures could be of great value to cholesterol reduction therapeutic accomplishments [25].

One of the potential probiotic mechanisms for cholesterol reduction exhibited by lactic acid bacteria is the deconjugation of bile salts. The high bile salt hydrolase (BSH) activity of \textit{Lactobacillus} spp. could exert a cholesterol-reducing effect that reduces cholesterol absorption and improves cholesterol catabolism, thus exerting beneficial effects against hypercholesterolemia [25].

\textit{L. plantarum} MA2 isolated from kefir was found to significantly reduce total cholesterol values in an in-vivo study in rats with a daily dose of 10\textsuperscript{11} cells per day per rat at the end of a five-week trial [26]. The genus \textit{Weissella} is different than \textit{Enterococcus} and \textit{Lactobacillus} and has cholesterol reduction activity in vitro and in-vivo [27]. The assimilation of cholesterol by \textit{Enterococcus faecalis} strain AG5 in an MRS medium containing sodium thioglycolate (with and without bile salt) was also studied by Misra et al. [28] and was found to be in the range of 16 to 53%. Hati et al. [29] mentioned that the culture of \textit{Lactobacillus fermentum} isolated from fermented rice-based beverages consumed by Khasi tribes of the Meghalaya state in India showed maximum cobalamin production after 36 h of incubation (0.05 µg/mL).

The hypocholesterolemic effect of LAB may be due to various mechanisms, including bile salt hydrolase activity, incorporating cholesterol into their membranes, binding cholesterol on their cell surfaces, and assimilation of cholesterol while growing. [6]. The isolates have shown BSH activity and a reduction in cholesterol in the growth medium; hence, the in vitro cholesterol reduction activity may be due to the BSH activity and could have augmented some other mechanisms that need to be studied further. Though Santos et al. [13] reported a five-fold increase in vitamin B\textsubscript{12} production by LAB in medium supplemented with glycero, in our study, glycerol supplementation in growth medium did not result in a significant increase in vitamin B\textsubscript{12} production by \textit{Enterococcus faecium}. Production of cobalamine has been reported in various species of LAB. The isolates obtained from traditionally produced yoghurt showed promising cholesterol reduction and vitamin B\textsubscript{12} production. Nami et al. [30] reported cholesterol reduction in growth medium by \textit{Enterococci} spp. isolated from fermented milk products. The range of cholesterol reduction varied from 33 to 72% in \textit{Enterococcus faecium}. Damodharan et al. [31], during studies on \textit{Lactobacillus helveticus} isolated from fermented cow milk, reported a 48% cholesterol reduction by the isolate. Aboseidah [32] investigated the culture of \textit{Enterococcus} isolated from infants’ stool, and found it to reduce cholesterol in the medium in the range of 48 to 71%. The results of the present study are in line with the earlier reports. Moreover, the culture isolates (except isolate 7MP) showed vitamin B\textsubscript{12} production and cholesterol reduction in the present study. This dual property of cholesterol reduction and vitamin B\textsubscript{12} production will add functionality to fermented foods such as yoghurt.

The study by Li et al. [33] reported 98 ng/mL production of vitamin B\textsubscript{12} by \textit{Lactobacillus} spp. using a combination of solid-phase extraction and the reverse-phase HPLC technique.
This is much higher than our finding, as we performed a bioassay to detect vitamin B\textsubscript{12} that estimates the bioactive principle of the compound.

Many probiotic LAB cultures have been studied as sources of vitamins other than vitamin B\textsubscript{12}, and Sabo et al. [34] reported the production of folate and riboflavin from \textit{L. lacti}. LAB are also reported to have the biosynthetic ability of vitamins other than vitamin B\textsubscript{12}; a complete riboflavin operon was found to be functioning in \textit{Bifidobacterium} species [35]. Since the LAB in the present study were isolated from traditionally produced yoghurt and their properties of HT-29 cell adhesion were studied, these cultures can offer these studied benefits to consumers [36].

Evidence of a PDU-CBI-cob-hem cluster (gene cluster involved in vitamin B\textsubscript{12} synthesis) has been reported in \textit{L. reuteri} DMS 20026, where genes are involved in vitamin B\textsubscript{12} production and the PDU locus codes for propanediol and glycerol dehydratase activity that needs vitamin B\textsubscript{12} as a cofactor [37]. Li et al. [38] reported vitamin B\textsubscript{12} production in \textit{Enterococcus} sp. and found 499 ng/mL by RP-HPLC. In addition, \textit{L. rossiae} encodes a complete biosynthetic pathway for vitamin B\textsubscript{12}. A co-fermentation study on vitamin B\textsubscript{12} production found that wheat bran fermented by \textit{P. freudenreichii} and \textit{L. brevis} can effectively yield vitamin B\textsubscript{12} to reduce cereal waste streams in the production of fortified plant-based food components [39]. Albano et al. [40] tested \textit{L. plantarum} and \textit{E. faecium} in cheese for their cholesterol reduction activity using modern techniques such as a GC-Flame Ionization Detector (GC-FID).

Glycerol has been regarded as a good and amply available carbon source to synthesize many essential products, including vitamins. Many members of \textit{Lactobacillus} spp. reduce glycerol and possess the mechanism for the synthesis of vitamin B\textsubscript{12} in a single chromosomal gene cluster. \textit{Lactobacillus} spp. harbor the genes responsible for glycerol utilization and vitamin B\textsubscript{12} production [41].

For identifying new isolates, 16S rRNA gene sequencing has become the preferred method because the 16S gene is highly conserved and variable in the genomic DNA for each species [42].

This study suggests the dual potential of isolates as a functional food with the potential for cholesterol reduction. However, detailed studies with the aid of advanced molecular techniques are required to claim the effects of this probiotic effect. There is also a necessity to characterize the medium for maximum production of vitamin B\textsubscript{12} if large-scale yield is under consideration for the additive application of isolated cultures. Since these are only in-vitro findings, the study itself poses limitations, too.

5. Conclusions

The results of this study revealed \textit{E. faecium} and \textit{L. pentosus}, which are isolated from yoghurt, as promising functional LAB cultures with in vitro cholesterol reduction ability and a source of vitamin B\textsubscript{12}. Since these isolates were also studied earlier for their other beneficial activities and primary probiotic properties, they could be used to develop functional food to alleviate the targeted conditions of hypercholesterolemia and vitamin B\textsubscript{12} deficiency. Thus, this finding opens the way to their use as candidates into novel functional foods to be tried for further detailed study. As the cultures are isolated from traditionally produced yoghurt and are adapted to the local environment and population, they can offer sustainable benefits. A further detailed study is needed to evaluate the mechanism underlying the cholesterol reduction ability. An in-vivo study is required to support the in-vitro findings to claim the beneficial effects.

\textit{Lactobacillus} spp. with a dual potential to produce vitamin B\textsubscript{12} and reduce cholesterol can serve as a good source of the vitamin while also catabolizing the excess lipid molecules. Such probiotics can be used to overcome vitamin B\textsubscript{12} deficiency and in the treatment of hypercholesterolemia.

The multifaceted use of probiotics as food additives increases their market value and attracts researchers to the improved formulation and proper dosing for the product user. However, the lack of clear understanding of cholesterol reduction mechanisms has created
an impediment in the administration of proper dosages and attainment of benefits. In this context, animal and human trials on hypocholesterolemic effects were performed by Ishimwe et al. [43]. Hence, the isolates have in-vitro functionality of vitamin fortification for the food that has now a days attracted the attention of many researchers of functional food.

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