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Cholesterol Reduction and Vitamin B_{12} 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 B_{12} 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 B_{12}. 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 B_{12}. 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 B_{12} fortification in fermented food.

Keywords: cholesterol reduction; *Enterococcus faecium*; *Lactobacillus pentosus*; vitamin B_{12} production; yoghurt

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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_{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_{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 *Enterococcus faecium* and *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) *E. faecium* (EF), (ii) *E. faecium* (Chole1), and (iii) *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_{12} auxotroph mutant culture of *E. coli* (Davis A mutant strain 113-3D) was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Vitamin B_{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^{10} cfu/mL was inoculated on BSH test medium (MRS medium supplemented with $CaCl_2$ and bile salt). The plates were incubated at 37 °C for 48h 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^{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× *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

$$\text{Cholesterol reduced } \left(\frac{\mu g}{mL}\right) = \text{Cholesterol inControl} - \text{Cholesterol in the test.}$$

2.3. Vitamin B_{12}Pro duction Assay

For this purpose, the log phase culture of each isolate was separately grown on vitamin B_{12} 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 B_{12} was considered its capacity to synthesize vitamin B_{12} [11].

2.3.1. Cells Extract Method

Having confirmed the ability of an organism to produce vitamin B_{12}, the cultures were subjected to cell lysis and extraction of vitamin B_{12}followed by an estimation of vitamin B_{12}. 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 B_{12} estimation.

2.3.2. Vitamin B_{12} Bioassay

A vitamin B_{12} bioassay was performed according to Madhu et al. [12] and Santos et al. [13], with some modifications. For the diffusion assay, vitamin B_{12} 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 B_{12} 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 B_{12} content in the sample was estimated as vitamin B_{12} produced per mL [12,13].

2.3.3. Influence of Glycerol on Vitamin B_{12} Production

To check the effect of glycerol on vitamin B_{12} production, a 0.5% (*v*/*v*) glycerol was added to the vitamin B_{12} growth medium. Cultures were grown in glycerol-amended medium at 37 °C for 24 h, followed by cell extraction and of vitamin B_{12} 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 B_{12} 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 forthe 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.

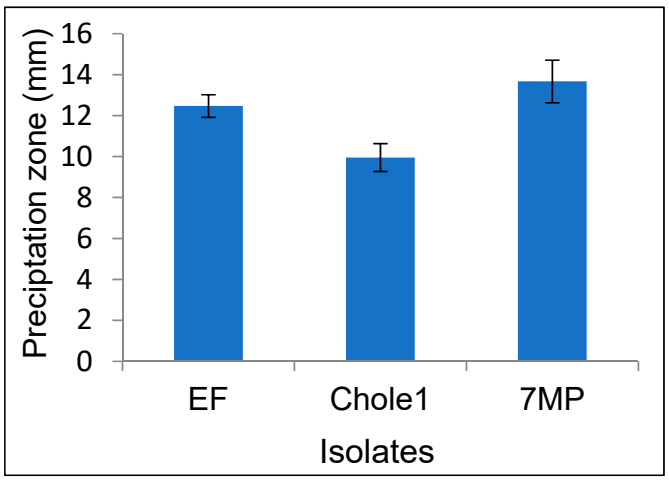

Figure 1. BSH activity of isolates.

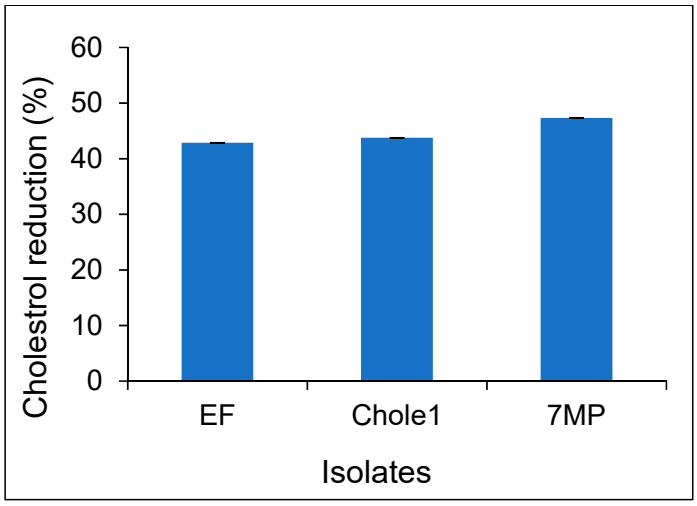

Figure 2. Reduction of cholesterol by the isolates.

3.2. Vitamin B_{12} Production

Good growth of the isolates *E. faecium* (EF) and *E. faecium* (Chole1) was observed on a vitamin B_{12}-free medium. This growth on the B_{12}-free medium indicated the vitamin B_{12}-producing abilities of the isolates *E. faecium* (EF) and *E. faecium* (Chole1). Both the isolates EF and Chole1 produced 1 ng/mL vitamin B_{12} (Figure 3), whereas the isolate *L. pentosus* (7MP) did not grow on the B_{12}-free medium. This indicated its inability to produce the vitamin B_{12} required for its growth, and hence it was considered a non-producer of vitamin B_{12}.

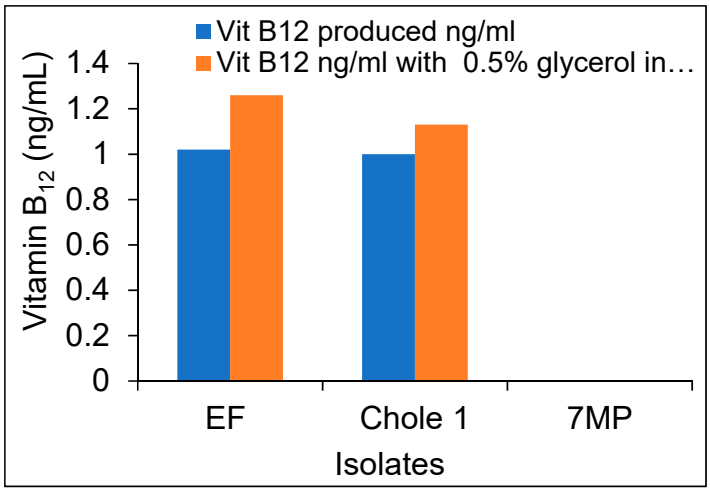

Figure 3. Vitamin B_{12} production by LAB isolates.

3.3. Influence of Glycerol on Vitamin B_{12} Production

Supplementation of glycerol in the growth medium resulted in a slight improvement in vitamin B_{12} production. This supplementation improved vitamin B_{12} 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

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 andKX886789 [8].

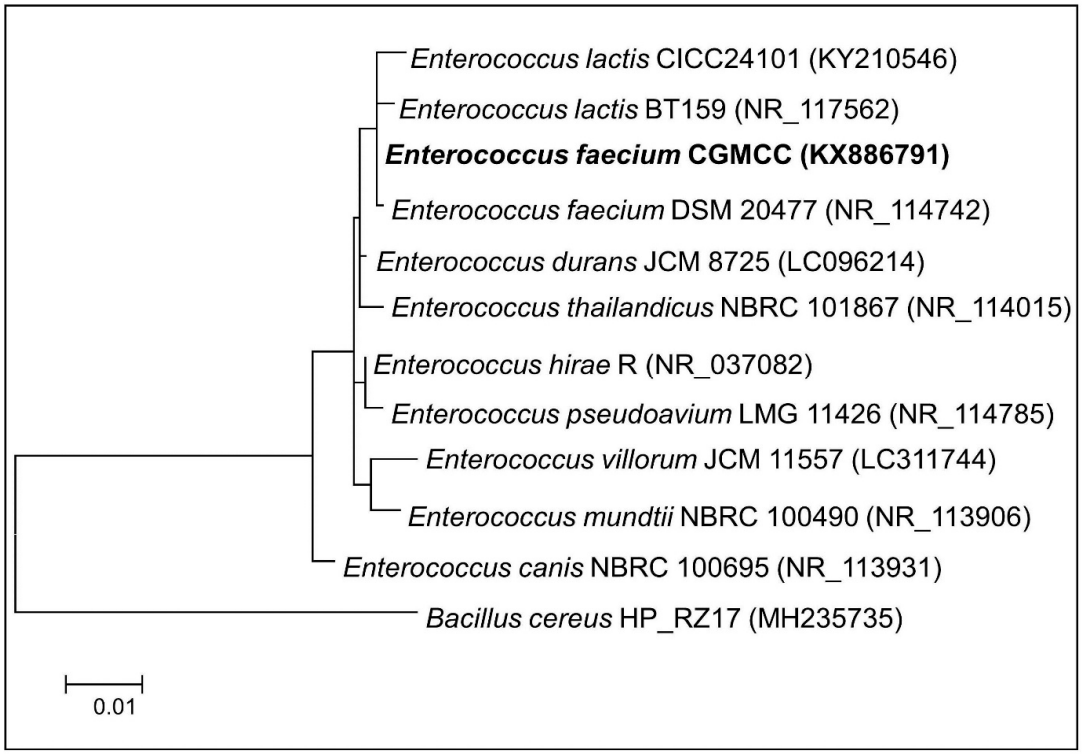

Figure 4. Phylogenetic tree of isolate Chole1.

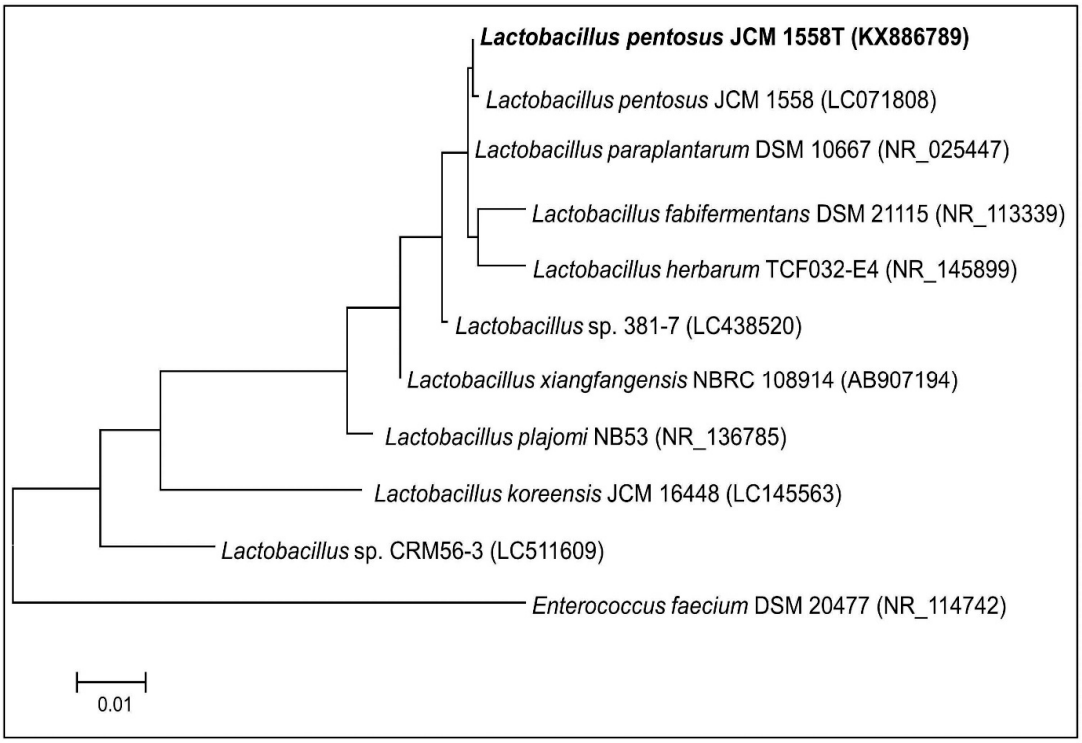

Figure 5. Phylogenetic tree of isolate 7MP.

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.

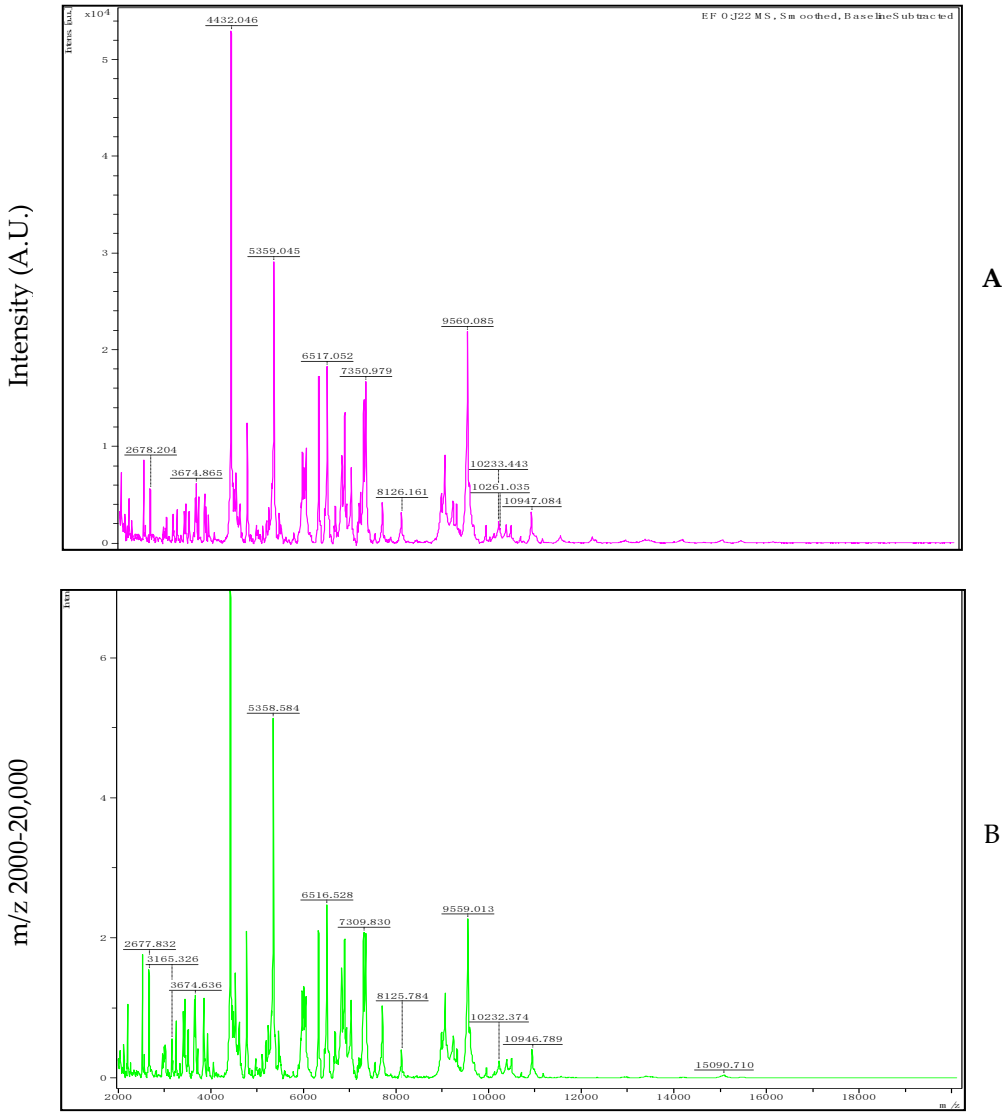

Figure 6. MALDI-TOF-MS spectra of isolate (**A**) EF and (**B**) Chole1.

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_{12} is a complex compound that is naturally produced by specific LAB. Thus, the enrichment of foods, particularly dairy foods, with vitamin B_{12} appears to be one of the best approaches to providing a good source of vitamin B_{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 *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 *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 *Lactobacillus* spp. could exert a cholesterol-reducing effect that reduces cholesterol absorption and improves cholesterol catabolism, thus exerting beneficial effects against hypercholesterolemia [25].

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^{11} cells per day per rat at the end of a five-week trial [26]. The genus *Weissella* is different than *Enterococcus* and *Lactobacillus* and has cholesterol reduction activity in vitro and in-vivo [27]. The assimilation of cholesterol by *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 *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_{12} production by LAB inmedium supplemented with glycerol, in our study, glycerol supplementation in growth medium did not result in a significant increase in vitamin B_{12} production by *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_{12} production. Nami et al. [30] reported cholesterol reduction in growth medium by *Enterococci* spp. isolated from fermented milk products. The range of cholesterol reduction varied from 33 to 72% in *Enterococcus faecium.* Damodharan et al. [31], during studies on *Lactobacillus helviticus* isolated from fermented cow milk, reported a 48% cholesterol reduction by the isolate. Aboseidah [32] investigated the culture of *Enterococcus* isolated from infants' stool, and found it to reduce cholesterol in the medium in the range of48 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_{12} production and cholesterol reduction in the present study. This dual property of cholesterol reduction and vitamin B_{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_{12} by *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_{12} that estimates the bioactive principle of the compound.

Many probiotic LAB cultures have been studied as sources of vitamins other than vitamin B_{12}, and Sabo et al. [34] reported the production of folate and riboflavin from *L. lacti.* LAB are also reported to have the biosynthetic ability of vitamins other than vitamin B_{12}; a complete riboflavin operon was found to be functioning in *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_{12} synthesis) has been reported in *L. reuteri* DMS 20026, where genes are involved in vitamin B_{12} production and the PDU locus codes for propanediol and glycerol dehydratase activity that needs vitamin B_{12} as a cofactor [37]. Li et al. [38] reported vitamin B_{12} production in *Enterococcus* sp. andfound 499 ng/mL by RP-HPLC. In addition, *L. rossiae* encodes a complete biosynthetic pathway for vitamin B_{12}. A co-fermentation study on vitamin B_{12} production found that wheat bran fermented by *P. freudenreichii* and *L. brevis* can effectively yield vitamin B_{12} to reduce cereal waste streams in the production of fortified plant-based food components [39]. Albano et al. [40] tested *L. plantarum* and *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 *Lactobacillus* spp. reduce glycerol and possess the mechanism for the synthesis of vitamin B_{12} in a single chromosomal gene cluster. *Lactobacillus* spp. harbor the genes responsible for glycerol utilization and vitamin B_{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_{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 *E. faecium* and *L. pentosus,* which are isolated from yoghurt, as promising functional LAB cultures with invitro cholesterol reduction ability and a source of vitamin B_{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_{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.

Lactobacillus spp. with a dual potential to produce vitamin B_{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_{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.

Author Contributions: Conceptualization: S.S.D., M.S.P. and R.A.W.; methodology: R.A.W.; formal analysis H.A.A.-S. and M.M.A.; writing—original draft: R.A.W.; writing—review and editing: R.Z.S., R.D. and S.D.; S.S.D. and M.S.P.; supervision, S.S.D. and M.S.P. Funding acquisition; A.M.E. All authors have read and agreed to the published version of the manuscript.

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References

1. Linares, D.M.; Gomez, C.; Renes, E.; Fresno, J.; Tornodijo, R.; Ross, R.P.; Stanton, C. Lactic acid bacteria and *Bifidobacteria* with potential to design natural biofunctional health-promoting dairy foods. *Front. Microbiol.* **2017**, *8*, 846. [CrossRef] [PubMed]
2. Zhao, W.; Liu, Y.; Latta, M.; Ma, W.; Wu, Z.; Chen, P. Probiotic database: A potential source of fermented foods. *Int. J. Food Prop.* **2019**, *221*, 198–217. [CrossRef]
3. Marco, M.L.; Heeney, D.; Binda, S.; Cifelli, C.J.; Cotter, P.D.; Foligné, B.; Gänzle, M.; Kort, R.; Pasin, G.; Pihlanto, A.; et al. Health benefits of fermented foods: Microbiota and beyond. *Curr. Opin. Biotechnol.* **2017**, *44*, 94–102. [CrossRef] [PubMed]
4. Kok, C.R.; Hutkins, R. Yoghurt and other fermented foods as sources of health-promoting bacteria. *Nutr. Rev.* **2018**, *76*, 4–15. [CrossRef] [PubMed]
5. Liu, Y.; Zhao, F.; Liu, J.; Wang, H.; Han, X.; Zhang, Y.; Yang, Z. Selection of cholesterol-lowering lactic acid bacteria and its effect on rats fed with high-cholesterol diet. *Curr. Microbiol.* **2017**, *74*, 623–631. [CrossRef]
6. Singhal, N.; Mayrya, A.K.; Mohanty, S.; Kumar, M.; Virdi, J.S. Evaluation of bile salt hydrolase, cholesterol-lowering capabilities, and probiotic potential of *Enterococcus faecium* isolated from the rhizosphere. *Front. Microbiol.* **2019**, *10*, 1567. [CrossRef]
7. Rizzo, G.; Lagana, A.S.; Maria, A.; Rapisarda, C.; Maria, G.; Ferrera, G.L.; Buscema, M.; Rossetti, P.; Nigro, A.; Mascia, V.; et al. Vitamin B12 among vegetarians: Status, assessment and supplementation. *Nutreints* **2016**, *8*, 767. [CrossRef]
8. Walhe, R.A.; Patole, M.S.; Diwanay, S.S. Some beneficial properties of bacterial isolates from traditionally fermented Indian foods. *Bionano Front.* **2017**, *10*, 120–126.
9. Kunzes, A.K.; Bhalla, T.C. In vitro cholesterol assimilation and functional enzymatic activities of putative probiotic *Lactobacillus* sp isolated from fermented foods/beverages of northwest India. *J. Nutr. Food Sci.* **2016**, *6*, 1–5.
10. Wu, C.C.; Weng, W.L.; Lai, W.L.; Tsai, H.P.; Liu, W.H.; Lee, M.H.; Tsai, Y.C. Effect of *Lactobacillus Plantarum* strain K21 on high-fat diet-fed obese mice. *Evid. Based Complement. Altern. Med.* **2015**. [CrossRef]
11. Masuda, M.; Ide, M.; Utsumi, H.; Niiro, T.; Shimamura, Y.; Murata, M. Production potency of folate, vitamin B12, and thiamine by lactic acid bacteria isolated from Japanese pickles. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 2061–2067. [CrossRef]
12. Madhu, A.N.; Giribhattanavar, P.; Narayam, M.S.; Prapulla, S.G. Probiotic lactic acid bacterium from *Kanjika* as a potential source of vitamin B12: Evidence from LC-MS, immunological and microbiological techniques. *Biotechnol. Lett.* **2009**. [CrossRef]
13. Santos, F.; Teusink, B.; Molenar, D.; Heck, M.; Wels, M.; Seiuwerts, S.; deVose, W.M. Effect of Amino Acid Availability on Vitamin B12 Production in *Lactobacillus reuteri*. *Appl. Environ. Microbiol.* **2009**, *75*, 3930–3936. [CrossRef]
14. Gangurde, N.S.; Sayyed, R.Z.; Kiran, S.; Gulati, A. Development of eco-friendly bioplastic like PHB by distillery effluent microorganisms. *Environ. Sci. Pollut. Res.* **2013**, *20*, 488–497. [CrossRef]
15. Sambrook, J.; Russel, D. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2001.

16. Pediyar, V.; Adam, K.A.; Badri, N.N.; Patole, M.; Shouche, Y.S. *Aeromonas culicicola* sp. nov., from the midgut of *Culexquinquefasciatus*. *Int. J. Syst.Evol. Microbiol.* **2002**, *52*, 1723–1728.
17. The National Center for Biotechnology Information. Available online: http://www.ncbi.nlm.nih.gov (accessed on 2 April 2021).
18. EzBioCloud Database. Available online: http://www.eztaxon.org (accessed on 2 April 2021).
19. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
20. Kimura, M. A simple method for estimating the evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
21. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
22. Rahi, P.; Prakash, O.; Shouche, Y.S. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass-Spectrometry (MALDI-TOF MS) Based Microbial Identifications: Challenges and Scopes for Microbial Ecologists. *Front. Microbiol.* **2016**, *7*, 1359. [CrossRef]
23. Ly, D.; Mayrhofer, S.; Yogeswara, B.; Nguyen, T.; Domig, K.J. Identification, classification, and screening for γ-Aminobutyric acid production in lactic acid bacteria from Cambodian fermented foods. *Biomolecules* **2019**, *9*, 768. [CrossRef]
24. Parker, R.E. Continuous distribution: Tests of significance. In *Introductory Statistics for Biology*, 2nd ed.; Arnold, B.S., Ed.; Cambridge University Press: Cambridge/London, UK, 1979; pp. 18–42.
25. Khare, A.; Gaur, S. Cholesterol-Lowering Effects of *Lactobacillus* Species. *Curr. Microbiol.* **2020**, *77*, 638–644. [CrossRef] [PubMed]
26. Slattery, C.; Cotter, P.D.; O'Toole, P.W. Analysis of Health Benefits Conferred by *Lactobacillus* Species from Kefir. *Nutrients* **2019**, *11*, 1252. [CrossRef]
27. Should, I.; Rehaiem, A.; Najjar, A.; Usai, D.; Boudabous, A.; Sechi, A.L.; Hadda-Imene, H. Functional Probiotic Assessment and In Vivo Cholesterol-Lowering Efficacy of *Weissella* sp. associated with Arid Lands Living-Hosts. *BioMed Res. Int.* **2018**. [CrossRef]
28. Misra, A.; Shravanti, S.K.; Ghosh, A.R. Probiotic *Enterococcus faecalis* AG5 effectively assimilates cholesterol and produces fatty acids, including propionate. *FEMS Microbiol. Lett.* **2019**, *366*, 1–9.
29. Hati, S.; Patel, M.; Mishra, B.K.; Das, S. Short-chain fatty acid and vitamin production potentials of Lactobacillus isolated from fermented foods of Khasi Tribes, Meghalaya, India. *Ann. Microbiol.* **2019**, *69*, 1191–1199. [CrossRef]
30. Nami, Y.; Vaseghi Bakhshayesh, R.; Mohammadzadeh Jalaly, H.; Lotfi, H.; Eslami, S.; Hejazi, M.A. Probiotic properties of *Enterococcus* isolated from artisanal dairy products. *Front. Microbiol.* **2019**, *10*, 300. [CrossRef] [PubMed]
31. Damodharan, K.; Palaniyandi, S.A.; Yang, S.H.; Suh, J.W. Functional probiotic characterization and in vivo cholesterol-lowering activity of *Lactobacillus helviticus* isolated from fermented cow milk. *J. Microbiol. Biotechnol.* **2016**, *26*, 1675–1686. [CrossRef]
32. Aboseidah, A.; Rasmey, A.M.; Osman, M.M.; Des Ouky, S.G.; Kamal, N. Cholesterol reduction in vitro by novel probiotic lactic acid bacterial strains of Enterococcus isolated from healthy infant's stool. *Afr. J. Microbiol. Res.* **2017**, *1138*, 1434–1444.
33. Li, P.; Gu, Q.; Yang, L.; Yu, Y.; Wang, Y. Characterization of extracellular vitamin B_{12} producing *Lactobacillus Plantarum* strains and assessment of the probiotic potentials. *Food Chem.* **2017**, *234*, 494–501. [CrossRef]
34. Sabo, S.S.; Mendes, M.A.; Araujo, E.S.; Muradian, L.B.; Makiyama, E.N.; Le Blancs, J.G.; Borelli, P.; Fock, R.A.; Knobl, T.; Oliveira, R.S. Bioprospecting of probiotics with antimicrobial activities against *Salmonella* Heidelberg produces B-complex vitamins as potential supplements in poultry nutrition. *Sci. Rep.* **2020**, *10*, 7235. [CrossRef]
35. Thakur, K.; Tomar, S.K.; De, S. Lactic acid bacteria as a cell factory for riboflavin production. *Microb. Biotechnol.* **2015**, *9*, 441–451. [CrossRef] [PubMed]
36. Walhe, R.A.; Patole, M.S.; Diwanay, S.S. Adhesion inhibition of *Salmonella enterica* sub-species; *typhimurium* to HT-29 cells by LAB isolates from traditionally fermented Indian foods. *Int. Res. J. Sci. Eng.* **2018**, *6*, 31–34.
37. De Angelis, M.; Bottacini, F.; Fosso, B.; Kelleher, P.; Calasso, M.; Di Cagno, R.; Ventura, M.; Picardi, E.; Van Sinderen, D.; Gobbetti, M. *Lactobacillus rossiae*, a Vitamin B12 Producer, Represents a Metabolically Versatile Species within the Genus *Lactobacillus*. *PLoS ONE* **2014**, *9*, 1–11. [CrossRef]
38. Li, P.; Gu, Q.; Wang, Y.; Yu, Y.; Yang, L.; Chen, J.V. Novel vitamin B_{12}-producing *Enterococcus* spp. and preliminary in vitro evaluation of probiotic potentials. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 6155–6164. [CrossRef] [PubMed]
39. Xie, C.; Coda, R.; Chamlagain, B.; Varmanen, P.; Piironen, V.; Katina, K. Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in Wheat Bran for in situ Production of Vitamin B12. *Front. Microbiol.* **2019**, *10*, 1541. [CrossRef]
40. Albano, C.; Morandi, S.; Silvetti, T.; Casiraghi, M.C.; Manini, F.; Brasca, M. Lactic acid bacteria with cholesterol-lowering properties for dairy applications: In vitro and situ activity. *J. Dairy Sci.* **2018**, *101*, 10807–10818. [CrossRef]
41. Santos, F.; Spinler, J.K.; Saulnier, D.M.A.; Molenaar, D.; Teusink, B.; de Vos, W.M.; Versalovic, J.; Hugenholtz, J. Functional identification in *Lactobacillus reuteri* of a PocR-like transcription factor regulating glycerol utilization and vitamin B_{12} synthesis. *Microb. Cell Fact.* **2011**, *10*, 55. [CrossRef]
42. Sonawane, M.S.; Chaudhary, R.D.; Shouche, Y.S.; Sayyed, R.Z. Insect gut bacteria: A novel source for siderophore production. *Proc. Natl. Acad. Sci. India Sect. Biol. Sci.* **2016**, *88*, 567–572. [CrossRef]
43. Ishimwe, N.; Daliri, E.B.; Lee, B.H.; Fang, F.; Du, G. The perspective on cholesterol-lowering mechanisms of probiotics. *Mol. Nutr. Food Res.* **2015**, *59*, 94–105. [CrossRef]