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Lactid Acid Bacteria (LAB) Selection for Vitamin B12 (Cobalamin) Synthesizer

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Vitamin B12 is an essential nutrient for humans and animals. The vitamin B12 content is high in animal meat, so vegetarians and vegans need to look for suitable sources of vitamin B12. Tempeh is one of the most common vitamin B12 sources in Indonesia. Tempeh is made from soybeans with *Rhizopus oligosporus*, but in the process, it was contaminated with a lot of bacteria (Radita *et al.* 2021). Bacteria such as lactic acid bacteria, *Klebsiella pneumoniae* and *Citrobacter freundii*, are organisms that can synthesize vitamin B12 (Keuth dan Bisping 1994). In this study, we isolated bacteria from tempeh and tested their ability to synthesize vitamin B12 with several existing bacterial collections. The research aim to obtain bacteria capable of synthesizing vitamin B12. This research includes isolation and characterization of LAB, screening of vitamin B12-synthesizing LAB, molecular analysis using 16S rRNA gene, and production of vitamin B12 using HPLC analysis. Ten isolates were analyzed for the ability to synthesize vitamin B12. Through its ability to grow on vitamin B12 assay media, eight isolates were obtained. Analysis of the 16S rRNA gene showed that the isolate from tempeh was closely related to *Lactiplantibacillus fermentum*. The results of HPLC analysis that five isolates can produce vit B12 but with a low concentration.

Keywords: tempeh, HPLC, *Lactiplantibacillus fermentum*

Vitamin B12 is also known as cobalamin. The structure of vitamin B12 has a core consisting of a corrine ring surrounding a cobalt ion in the middle. Four nitrogen atoms are formed from 5,6-imethylbenzimidazole ribonucleotide, which are below the plane of the corin ring and the variable group (R) is above the plane of the corin ring. The variable group can be occupied by several ligands, such as hydroxyl, cyano, methyl, or 5'-deoxadenosyl groups (Nielsen *et al.* 2012). Vitamin B12 prevents animal pernicious anemia (Fang *et al.* 2017) and is an essential nutrient for humans and animals (Balabanova *et al.* 2021). Vitamin B12 has various biochemical functions, such as DNA synthesis and regulation, fatty acid degradation, and amino acid metabolism (Bernhardt *et al.* 2019). The natural forms of vitamin B12 (MeCbl and AdoCbl) are only synthesized by bacteria, which are required as essential cofactors for the cytosolic enzyme methionine synthase (methionine formation) and the formation of succinyl-CoA in human and animal metabolism (Froese *et al.* 2019). The function of cytosolic methionine synthase is to catalyze the methylation of homocysteine to methionine, while the function of methylmalonyl-CoA mutase is to convert methylmalonyl-CoA to succinylCoA in the mitochondria. The previous reaction is related to folate metabolism by converting the group of 5-methyl tetrahydrofolate to tetrahydrofolate.

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Tetrahydrofolate is essential for the production of purines and pyrimidines. Vitamin B12 plays a vital role in the human body, and its deficiency can cause megaloblastic anemia, peripheral arterial disease, and various neurological disorders (Nielsen *et al.* 2012). Peripheral arterial disease can trigger the risk of hypertension, diabetes mellitus, and dyslipidemia (Zsóri *et al.* 2013).

Organisms that can synthesize vitamin B12 are prokaryotes, especially bacteria. Bacteria capable of synthesizing cobalamin are *Acetobacter pasteurianus*, *Propionibacterium freudenreichii* ssp. *freudenreichii*, *Propionibacterium freudenreichii* ssp. *shermanii*, and *Pseudomonas denitrificans*. *Propionibacterium freudenreichii* ssp. *shermanii* and *Pseudomonas denitrificans* are often used in the food industry because of their high vitamin B12 productivity and fast growth; these bacteria have GRAS (Generally Recognized as Safe) status from the United States Food and Drug Administration (Bernhardt *et al.* 2019). Other bacteria such as *Pediococcus pentosaceus* isolated from traditional fermented foods in India and including lactic acid bacteria (LAB) are capable of producing vitamin B12 and have the ability to reduce cholesterol (Rajan *et al.* 2021). LAB isolated from yogurt, which is *Enterococcus faecium*, can also produce 1 ng/mL of vitamin B12 (Walhe *et al.* 2021). Apart from having a role in health, LAB also includes bacteria with GRAS status (Iyer *et al.* 2013), so LAB can be used as vitamin B12 synthesizing bacteria. The bacteria *Klebsiella pneumoniae* IIEMP-3 was also found to produce vitamin B12 in tempeh (Yulandi *et al.* 2016), the vitamin B12 content in soybean tempeh ranges from 0.7 µg/100 g in fresh tempeh to 8 µg/100 g during storage (Wolkers–Rooijackers *et al.* 2018).

High levels of vitamin B12 can be found in meat, milk, and fish. Vitamin B12 levels in beef are 1.0-2.0 µg/100 g wet weight, while in chicken, it is 0.5 µg/100 g wet weight. The level of vitamin B12 in sheep's milk is 0.71 µg/100 g of milk, in cow's milk it is 0.35 µg/100 g of milk, and in goat's milk it is 0.06 µg/100 g of milk. Chicken eggs contain vitamin B12 as much as 0.9 µg/100 g wet weight, mainly found in the egg yolk (Watanabe dan Bito 2018). The vitamin B12 content in the fish body, except the head and bones, is 5.1 µg per fish body (Nishioka *et al.* 2011). The vitamin B12 content in edible shellfish is 60 µg/100 g wet weight, while in edible snails it is 20 µg/100 g wet weight (Tanioka *et al.* 2014). The vitamin B12 content is very high compared to the vitamin B12 content in seaweed, which is 0.2-0.5 µg/100 g wet weight. The vitamin B12 content in vegetables such as spinach is deficient at 6.9 ng/g wet weight, and in soybeans 1.6 ng/g wet weight (Watanabe dan Bito 2018). The fruiting bodies of mushrooms contain varying amounts of vitamin B12, depending on the type. Oyster mushrooms have a vitamin B12 content of 0.01-0.09 µg/100 g wet weight (Watanabe *et al.* 2012). The highest vitamin B12 content is in shiitake, namely 5.6 µg/100 g wet weight, but vitamin B12 in shiitake is a vitamin B12 which is inactive in the human body, so it cannot be absorbed by the body (Bito *et al.* 2014).

The low vitamin B12 content in vegetables affects vegetarians. The average intake of vitamin B12 obtained by vegetarians is around 0.4 µg/day (Bernhardt *et al.* 2019), this does not meet the standard daily intake of vitamin B12. Ideally, women get 3.47 µg/day of vitamin B12, while men get 4.18 µg/day (Brouwer-Brolsma *et al.* 2015). Based on this, it is necessary to research bacteria that produce vitamin B12 because the natural form of vitamin B12 is only synthesized by bacteria, apart from that, bacteria can also be used as a supplement in food. The bacteria used in this research were lactic acid bacteria (LAB) isolated from tempeh because the vitamin B12 content in tempeh is higher than the vitamin B12 content in other vegetable foods. Apart from that, LAB isolates *Pediococcus pentosaceus*, *Lactiplantibacillus plantarum*, *Lactobacillus pentosus*, and P*ediococcus pentosaceus* which are from the collection of the Microbial Bioprospection Laboratory were also used. These isolates have antimicrobial activity, are sensitive to antibiotics, and have the ability to inhibit mold growth (Rosyidah 2013; Erdiandini *et al.* 2015; Tsaaqifah 2017; Turnip *et al.* 2018; Karyawati 2019; Riani *et al.* 2020).

MATERIAL AND METHODS

Isolation and Purification of LAB. Lactic acid bacteria were isolated from tempeh aseptically. Five grams of tempeh was put into 45 mL of sterile 0.85% (w/v) NaCl solution and homogenized. Samples were diluted using a graded dilution method ranging from 10^{-1} to 10^{-7}. Samples were taken from 10^{-5} to 10^{-7} dilutions of 1 mL, then grown on MRS agar media containing 1% calcium carbonate ($CaCO_3$). Samples were incubated for 48 hours at 37°C. The bacteria obtained were tested for clear zones using MRS agar media containing 1% $CaCO_3$, then the resulting clear zone was observed to ensure that the colonies selected were LAB. The sample was purified to obtain a single isolate, then characterized by Gram staining and catalase testing (Barus *et al.* 2020).

BAL Rejuvenation Microbial Bioprospection Laboratory Collection. BAL rejuvenation refers to Aprisal *et al.* (2020), which has been modified. Lyophilized LAB isolates were grown in 5 mL MRS-Broth media and then incubated for 24 hours at 37°C. After the bacteria grow, subculture is carried out in 15 mL MRS broth media. Samples were incubated for 24 hours at 37°C. The bacterial subculture was rejuvenated and tested for clear zones using MRS agar media containing 1% $CaCO_3$, incubated for 24 hours at 37°C, then the resulting clear zone was observed to ensure that the isolate was LAB. Next, the samples were characterized by Gram staining and catalase test. The following is a list of isolates used (table 1).

Table 1 List of BAL isolates from the Microbial Bioprospecting Laboratory collection.

Isolate code	Species
E1222	*Pediococcus pentosaceus*
E2211	*Pediococcus pentosaceus*
H2.34	*Lactiplantibacillus plantarum*
MA15	*Enterococcus faecium*
NHC8	*Lactobacillus pentosus*
NHC9	*Lactiplantibacillus plantarum*

BAL Screening for Vitamin B12 Synthesizers. Bacterial screening refers to Bernhardt *et al.* (2019), which has been modified. It was carried out using Himedia vitamin B12 assay broth media with the addition of 2.4% agar. Bacteria were grown in the media and then incubated for 24 hours at 37°C. Bacteria that successfully grew were re-inoculated into vitamin B12 assay broth media with the addition of 2.4% agar and then incubated for 24 hours at 37 °C. This step was carried out three times. Isolates that survived this test indicated their ability to synthesize vitamin B12.

Growth Curve. The selected LAB isolates were grown in 10 mL of MRS Broth media for pre-culture. Samples were incubated for 24 hours at 37°C, 1 mL of the culture that had grown was taken and placed in 9 mL of vitamin B12 assay broth (Kang *et al.* 2020). The growth curve was carried out using the TPC (Total Plate Count) method on MRS agar media for 36 hours, samples were taken every 6 hours. TPC results are calculated using the following formula (Parker *et al.* 2016):

$$CFU/mL = \frac{Number\ of\ colonies\ growing\ \times\ Dilution\ factor}{Volume\ inoculum}$$

DNA Extraction, Amplification, and Sequencing of the 16S rRNA Gene. LAB isolates were grown in MRS broth media and incubated for 24 hours at 37 °C. DNA was extracted using the Presto™ Mini gDNA Bacteria Kit. The concentration of DNA was measured using a nanodrop.

Amplification of the 16S rRNA gene was carried out using the MyTaq HS Red Mix PCR kit. The primers used are 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T). The PCR cycle begins with pre-denaturation for five minutes at 95°C, denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C, elongation for one minute at 72°C, and post-elongation for 15 minutes at 72 °C. PCR cycles were carried out 30x (Liew *et al.* 2009). PCR results were visualized on a 1% agarose gel using gel doc. 16S rRNA gene sequencing was carried out using Genetic Science services. Sequencing results were analyzed using NCBI BLASTn and phylogenetic tree construction using MEGA11

HPLC Vitamin B12. LAB isolates were grown in 50 mL of vitamin B12 assay broth. Cultures were incubated for 24 hours at 37°C. The bacterial cultures that had grown were harvested using a centrifuge (10,000 x*g*, 15 minutes), and the pellets obtained were washed using 20 mL of 0.2 M potassium phosphate buffer (pH 5.5). The sample was centrifuged again (10,000 ×g, 15 min) and resuspended in 1 mL of 0.2 M potassium phosphate buffer (pH 5.5) containing 0.1% potassium cyanide. The samples were vortexed, sterilized for 15 minutes at 121°C, vortexed again, and centrifuged (10,000 × g, 15 minutes). The supernatant was filtered using a 0.45 µm membrane filter and put into a microtube (Hugenschmidt *et al.* 2010).

HPLC was carried out using a C18 reversed-phase column, the detector was L-7455 DAD. The required elution conditions are 0 to 5 minutes linear gradient from 100% Milli-Q to 5% (v/v) acetonitrile solution, 5 to 7 minutes linear gradient from 5 to 15% (v/v) acetonitrile solution, 7 to 14 minutes gradient linear gradient from 15 to 20% (v/v) acetonitrile solution, 14 to 17 minutes linear gradient from 20 to 100% (v/v) acetonitrile solution, 17 to 20 minutes linear gradient from 100% acetonitrile solution to 100% Milli-Q, and 20 to 25 minutes 100% milliQ linear gradient. UV detection was carried out at a wavelength of 358 nm. The solution rate was set to 1.4 mL/min. The oven tempehrature was 30°C and the injection volume was 40 µL (Hugenschmidt *et al.* 2010).

RESULT

Isolation and Characterization LAB. The number of bacteria obtained from the isolation was 16 isolates, then tested for the clear zone. The number of bacteria that produced a clear zone was five isolates. The five isolates were coded 5.1.1; 5.1.2; 5.2.1; 5.2.2; and 6.1.1. These bacteria have morphological characteristics, which include a round colony shape, a convex colony surface shape, cream-colored colonies, are included in Gram-positive bacteria, do not produce the catalase enzyme, and have a basil cell shape. This is different from isolate 5.2.2, which is included in Gram-negative bacteria (table 2). The results of BAL rejuvenation from the Microbial Bioprospection Laboratory collection also produced a clear zone. Morphological characters include round colony shape, convex colony surface, cream colony color, included in Gram-positive bacteria, do not produce catalase enzymes, and bacillary shaped cells for isolates H2.34, NHC8, and NHC9. Isolates E1222, E2211, and MA15 were coccus (table 2). The total number of lactic acid bacteria obtained and used for further tests was ten isolates.

Table 2 Characteristics of lactic acid bacteria

Isolate code	Bacteria Gram	Catalase assay	Bacteria cell shape
5.1.1	Positive	Negative	Basil
5.1.2	Positive	Negative	Basil
5.2.1	Positive	Negative	Basil
5.2.2	Negative	Negative	Basil
6.1.1	Positive	Negative	Basil
H2.34	Positive	Negative	Basil
NHC8	Positive	Negative	Basil
NHC9	Positive	Negative	Basil
E1222	Positive	Negative	Coccus
E2211	Positive	Negative	Coccus
MA15	Positive	Negative	Coccus

BAL Screening for Vitamin B12 Synthesizers. Isolates that are thought to be able to synthesize vitamin B12 can be seen from their growth on vitamin B12 assay agar media (table 3). There were eight isolates of lactic acid bacteria, which were thought to be able to synthesize vitamin B12, this was because these eight isolates grew well on the screening media. The eight isolates were 5.1.1, 5.1.2, 5.2.1, 6.1.1, E1222, NHC8, NHC9, and H2.34. Isolate E2211 could not grow on the 1st, 2nd and 3rd streaks. The growth of the MA15 isolate on the screening media was unstable because it only grew slightly on the 1st and 3rd streaks, while it did not grow on the 2nd streak. Based on this, isolates E2211 and MA15 were deemed unable to synthesize vitamin B12.

Growth Curve of Lactic Acid Bacteria. Eight bacterial isolates had different exponential phases (table 4). Isolates E1222, NHC9, and 5.1.1 reached the peak of the exponential phase at 12th hour. Isolates NHC8, 5.1.2, and 5.2.1 reached the peak of the exponential phase at 18th hour. Isolates H2.34 and 6.1.1 reached the peak of the exponential phase at the 36th hour, although the number of colonies decreased at the 18th hour, in the following hour the number of colonies still increased until the 36th hour.

16S rRNA Gene Sequencing. Only lactic acid bacteria isolated from tempeh had their 16S rRNA gene sequenced. This is because the other test isolates had their 16S rRNA gene sequenced, and their species were known in previous research. The 16S rRNA gene analysis results on BLASTn NCBI showed that the four isolates isolated from tempeh had a 99% similarity level to the *Limosilactobacillus fermentum* species. This was confirmed by the phylogenetic tree (figure 1), which showed that the four isolates isolated from tempeh were closely related to the *Limosilactobacillus fermentum* species.

Table 3 Results of screening of vitamin B12 synthesizing LAB on vitamin B12 assay agar media

Isolate code	First streak	Second streak	Third streak
5.1.1	Growth	Growth	Growth
5.1.2	Growth	Growth	Growth
5.2.1	Growth	Growth	Growth
6.1.1	Growth	Growth	Growth
H2.34	Growth	Growth	Growth
NHC8	Growth	Growth	Growth
NHC9	Growth	Growth	Growth
E1222	Grow a little	Growth	Growth
E2211	Not growing	Not growing	Not growing
MA15	Grow a little	Not growing	Grow a little

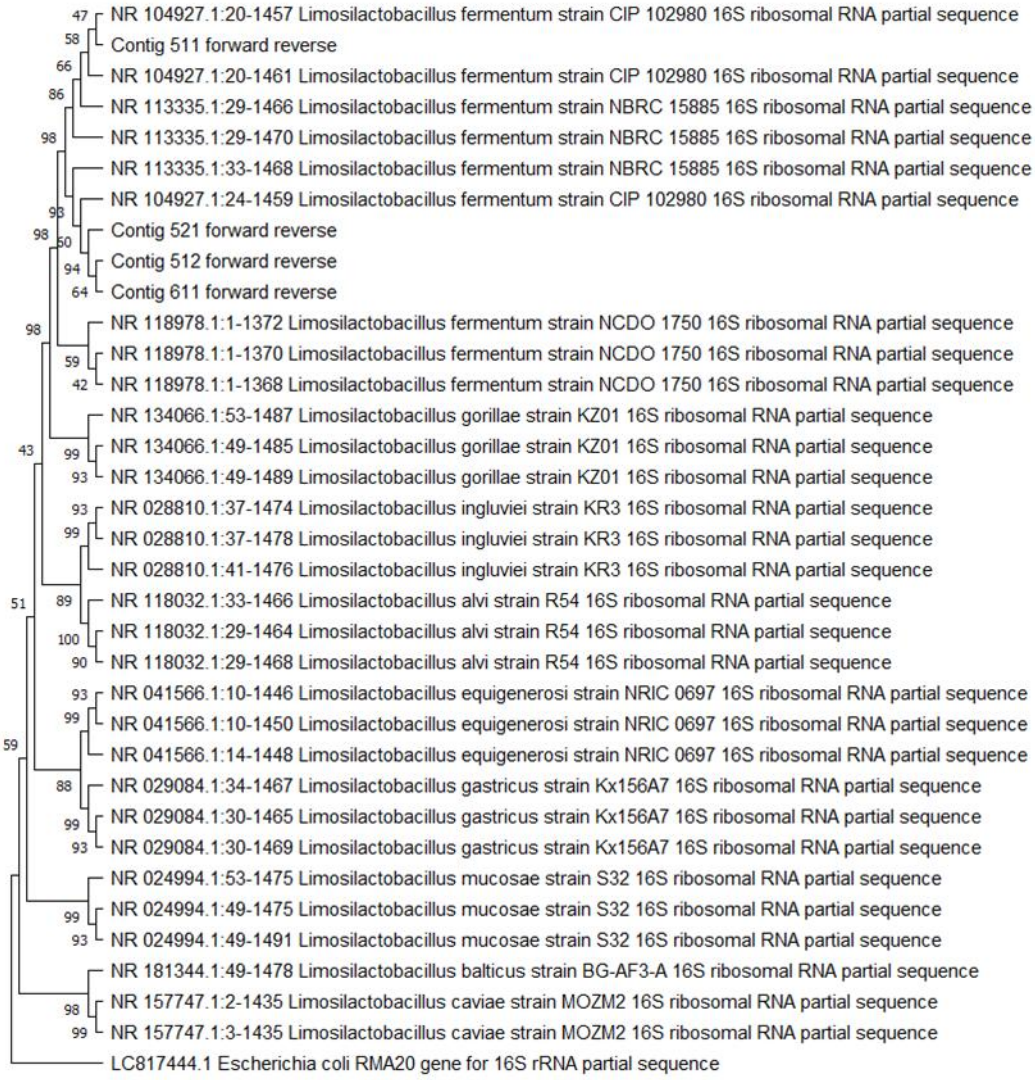

Figure 1. 16S rRNA phylogenetic tree isolate from tempeh

Table 4 Results of LAB growth on vitamin B12 assay medium

Isolate	Total coloni (log CFU/mL) hour to-						
	0	6	12	18	24	30	36
E1222	7,38 + 0,06	7,59 + 0,34	8,67 + 1,03	7,73 + 0,08	7,59 + 0,08	8,29 + 0,05	7,60 + 0,01
H2.34	7,41 + 0,01	8,08 + 0,06	8,40 + 0,41	8,37 + 0,49	8,75 + 0,49	8,54 + 0,39	8,76 + 1,70
NHC8	7,18 + 0,02	7,82 + 0,04	7,79 + 0,07	8,10 + 0,59	7,51 + 0,01	7,65 + 0,06	7,34 + 0,01
NHC9	7,20 + 0,05	8,20 + 0,10	8,61 + 0,35	8,39 + 0,06	8,44 + 0,15	8,55 + 0,25	8,48 + 1,07
5.1.1	7,28 + 0	8,12 + 0,06	8,63 + 0,18	8,16 + 0,53	7,26 + 0,02	6,60 + 0,04	6 + 0
5.1.2	7,30 + 0,01	8,12 + 0,35	8,30 + 0,08	8,62 + 0,74	7,53 + 0,02	6 + 0	6,30 + 0
5.2.1	7,11 + 0,04	6,78 + 0	7,99 + 0,40	8,28 + 0,31	7,61 + 0,51	7,72 + 0,24	7,11 + 0
6.1.1	7,08 + 0,02	6,78 + 0,02	7,93 + 0,12	7,88 + 0,40	7,68 + 0,06	7,73 + 0,20	8 + 0,18

HPLC Vitamin B12. Based on the standard HPLC chromatogram results (figure 2), the standard peak of vitamin B12 appeared at the 10th minute retention time. In samples 5.1.1, 5.1.2, 5.2.1, H2.34, and NHC9 a peak appeared at the 10th minute retention time, but the peak was very low. In samples 6.1.1, E1222, and NHC8 the peak appeared at the retention time of 9.7 minutes. A very low peak and a sample retention time that is different from the standard indicates that the sample does not contain vitamin B12.

DISCUSSION

The results of BAL from tempeh and from Microbial Bioprospection Laboratory collection also produced a clear zone. This is because the bacteria produce lactic acid, which causes the pH around the bacteria to drop so that the calcium carbonate in the media dissolves and produces a clear zone (Hasbi *et al*., 2024).

Isolate 5.2.2 is a Gram-negative bacterium but can produce lactic acid because it produces a clear zone when grown on MRS agar media containing 1% $CaCO_3$. According to (Lee *et al.* 2006), Gram-negative bacteria such as *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* produce lactic acid as a secondary metabolite. Isolate 5.2.2 not included in BAL, this is because BAL is a Gram-positive bacteria with the form of cocci or bacillus cells (Wang *et al.* 2021). Other literature also explains that the characteristics of LAB do not produce catalase enzymes, the colony color is cream, the colony shape is round, and the colony surface is convex (Rahmawati *et al.* 2021). The function of the catalase enzyme is to decompose hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). When hydrogen peroxide is dropped on bacteria that produce the enzyme catalase, it will produce oxygen bubbles which are seen as a positive result in the catalase test (Prastujati *et al.* 2022) Isolates E2211 and MA15 were deemed unable to synthesize vitamin B12, because they can't grow in vitamin B12 assay medium. According to Bernhardt *et al.* (2019) vitamin B12 medium assay is free from vitamin B12, so bacteria that can grow on vitamin B12 test media indicate their ability to synthesize vitamin B12. The differences in the exponential phase in the eight isolates could be caused by the different growth abilities of each individual bacteria (Yang *et al.* 2018).

The 16S rRNA gene analysis results showed that the four isolates isolated from tempeh had a 99% similarity level to the *Limosilactobacillus fermentum* species. Based on previous research looking at the diversity of bacteria in tempeh, the bacteria that dominates tempeh is *Limosilactobacillus fermentum* (Radita *et al.* 2017).

A very low peak and a sample retention time that is different from the standard indicates that the sample does not contain vitamin B12. This could be caused by the standard concentration being too high while the concentration of vitamin B12 in the sample is low. In addition, because the sample has not been purified, it still contains many other compounds, making it difficult to detect the target compound (Wiley 2003).

Eight isolates are thought to be able to synthesize vitamin B12. In the HPLC test, the peak of the standard retention time for vitamin B12 appeared at 10 minutes. In samples 5.1.1, 5.1.2, 5.2.1, H2.34, and NHC9 a peak appeared at the 10th minute retention time, but the peak was very low. The 16S rRNA gene analysis showed that the isolate isolated from tempeh was closely related to *Lactiplantibacillus fermentum*.

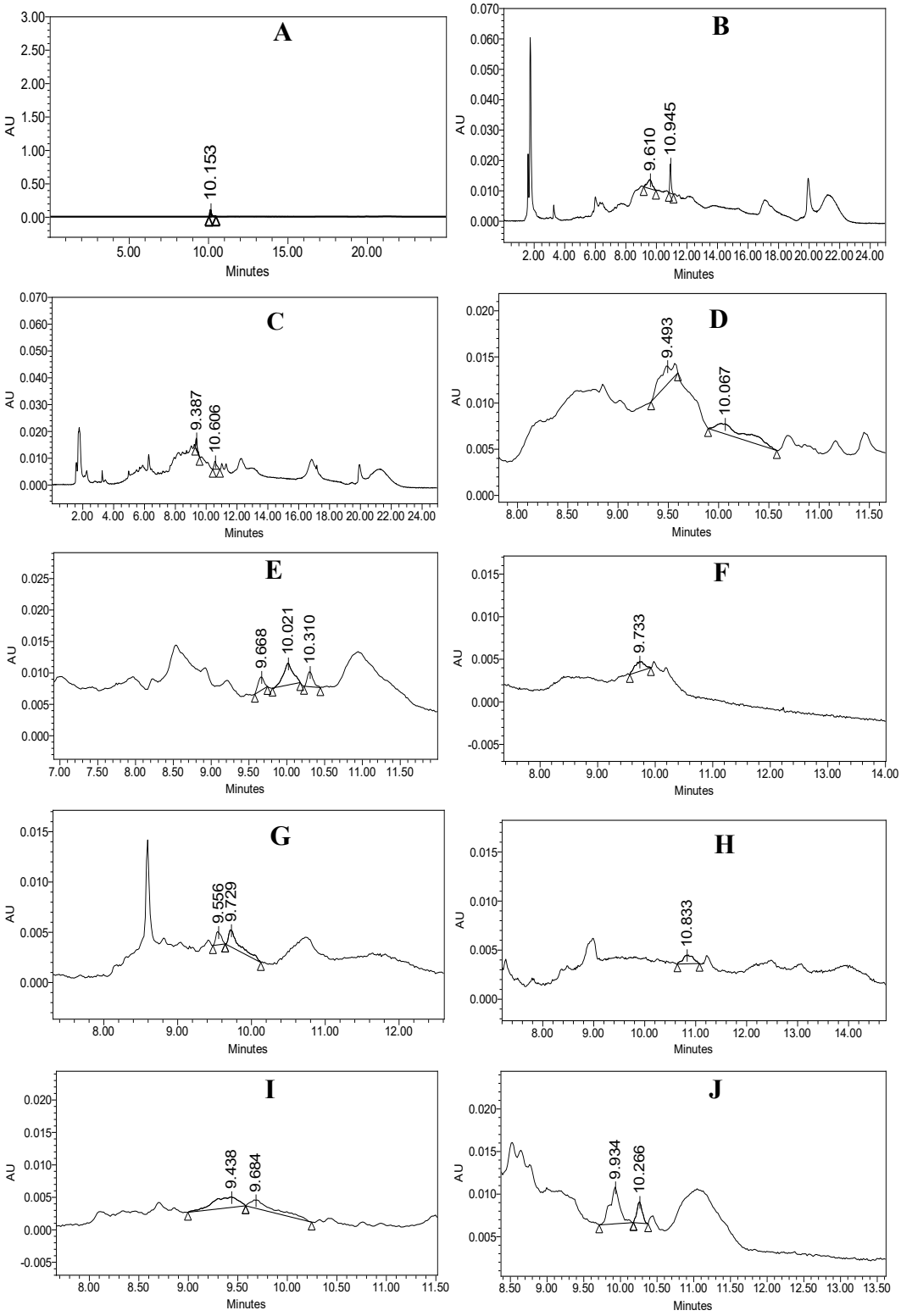

Figure 2 Vitamin B12 HPLC chromatogram. A) standard 25 ppm, B) control negative, C) isolate 5.1.1, D) isolate 5.1.2, E) isolate 5.2.1, F) isolate 6.1.1, G) isolate E1222, H) isolate H2.34, I) isolate NHC8, dan J) isolate NHC9

CONCLUSION

This study found lactic acid bacteria (LAB) from tempeh and laboratory collections that can produce vitamin B12. Eight out of ten LAB isolates grew on vitamin B12 assay media, showing their ability to synthesize this vitamin. Molecular analysis revealed that LAB from tempeh is closely related *to Limosilactobacillus fermentum*. HPLC analysis confirmed vitamin B12 production in five isolates, although the amounts were low. These results suggest that LAB associated with tempeh, especially *Limosilactobacillus fermentum*, may serve as vitamin B12-producing bacteria. Further work is needed to improve vitamin B12 production for functional food uses.

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