

RESEARCH ARTICLE

## Capability of *Lactobacillus reuteri* to Produce an Active Form of Vitamin B12 under Optimized Fermentation Conditions

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### Abstract

There has been considerable debate on the ability of *Lactobacillus reuteri* to produce the active form of vitamin B12. In this study, we demonstrated the ability of two wild type strains of *Lactobacillus reuteri* to produce  $\alpha$ -(5,6-dimethylbenzimidazolyl)-cobamidcyanide or cyanocobalamin which are the active form of vitamin B12. This was possible by supplying the required compounds of vitamin B12,  $\delta$ -aminolevulinic acid (ALA) and 5,6-dimethylbenzimidazole (DMB) under the appropriate conditions for bacteria to induce and complete the formation of vitamin B12 with DMB as the lower (Co $\alpha$ ) ligand. It was remarkable to find a microorganism possessing probiotic properties along with the capability to produce an active vitamin B12. High performance liquid chromatography (HPLC) coupled with mass spectrometry were used to identify vitamin B12 forms and confirm our results.

**Keywords:** *Lactobacillus reuteri*, cyanocobalamin, vitamin B12,  $\delta$ -aminolevulinic acid, probiotic properties.

### Introduction

Vitamin B12 is a water soluble vitamin and one of the largest complex vitamins with a molecular weight of over 1000. It consists of a corrin ring made up of four heterocyclic aromatic with cobalt at the center of the ring, which explains its scientific name, cobalamin. It is unique amongst the vitamins that it is only made by certain bacteria and synthesized through the involvement of approximately 30 enzyme mediated steps. Neither fungi, plants nor animals are capable of producing vitamin B12. Only bacteria and archaea have the enzymes required for its synthesis (Warren *et al.*, 2002). Vitamin B12 is used as a food supplement and is particularly important in the treatment of pernicious anemia. In addition, it plays an important role in the normal functioning of the nervous system and for the formation of blood. As well as, it is involved in the DNA synthesis and regulation, fatty acid synthesis and energy production (Stabler, 2012). In nature, the predominant form of vitamin B12 is adenosylcobalamin in eukaryotes especially exist in the mitochondria. It acts as cofactor for the enzyme methylmalonyl CoA mutase. The other major natural form of vitamin B12 is methylcobalamin. This is the predominant form in the cytosol that acts as cofactor for the enzyme methionine synthase. There is also hydroxocobalamin, resulting from the conversion of adenosylcobalamin and methylcobalamin by exposure to light. All forms of cobalamin are converted to cyanocobalamin to use for pharmacological and commercial purposes, because it is the most stable form of the vitamin B12 in the presence of light (Huang *et al.*, 1994; Mancina *et al.*, 1996; Raux *et al.*, 2000).

Furthermore, a great variety of vitamin B12 analogues which share a structural architecture consisting of a corrin ring with a cobalt ion chelated at the core such as pseudovitamin B12 (Santos *et al.*, 2007; Zemleni *et al.*, 2007; Taga and Walker, 2008). This molecule differs from cobalamin in  $\alpha$ -ligand, where it has adenine instead of 5,6-dimethylbenzimidazole bound in a  $\alpha$ -glycosidic link to C-1 of ribose and this because of the lack of synthesis oxygen to allow the transformation of riboflavin into DMB (Scott and Roessner, 2007). Pseudovitamin B12 is defined as biologically inactive in humans due to the 500-fold-lower binding affinity of pseudovitamin-B12 for the human B12 receptor (intrinsic factor) than vitamin B12 and this is largely contributed to the inability of humans to use it (Stupperich and Nexø, 1991).

Many food sources and supplements have proven to contain pseudovitamin-B12. Several reports have recently shown that pseudovitamin-B12 is the dominant corrinoid produced by the anaerobes *Clostridium cochlearium*, the cyanobacterium *Nostoc commune*, *Aphanizomenon flos-aquae*, the alga *Aphanothece sacrum* and *Lactobacillus reuteri* (Taranto *et al.*, 2003; Santos *et al.*, 2007). *Lactobacillus reuteri* is a Gram-positive, heterofermentative lactic acid bacteria (Kelleher and Broin, 1991) and a member of the gastrointestinal ecological systems of humans and other animals. This bacterium possesses certain probiotic properties, including lowering blood cholesterol levels in mice and stimulating anti-inflammatory activity in human cell lines.

In addition, *L. reuteri* possesses the unique ability to produce and excrete bacteriocin, reuterin (a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde [3-HPA]). The synthesis of reuterin is mediated through a cobalamin-dependent glycerol dehydratase, which catalyses the conversion of glycerol to 3-HPA (Talarico *et al.*, 1988; Talarico and Dobrogosz, 1989). The ability of *L. reuteri* has been demonstrated to utilize glycerol during glucose fermentation without exogenous cobalamin in a vitamin B12-free medium. Thus, this indicated the ability of *L. reuteri* to produce cobalamin by itself and capable of meeting the auxotrophic B12 requirements (Taranto *et al.*, 2003). Moreover, the enzymatic machinery necessary for the anaerobic B12 biosynthesis pathway has been identified to encode in the chromosome of *L. reuteri* (Santos *et al.*, 2008). This is unlike most of lactic acid bacteria (LAB) species which are auxotrophic for vitamin B12 (Morishita *et al.*, 1981). However, there has been considerable controversy about the ability of *L. reuteri* to produce pseudo-B12 instead of vitamin B12 that has already proven that pseudovitamin-B12 is the corrinoid produced by *L. reuteri* under anaerobic conditions (Santos *et al.*, 2007). In this study, using HPLC and mass spectrometry we have demonstrated the ability of *L. reuteri* to produce an active vitamin B12 by optimizing the fermentation conditions after studying the entire vitamin B12 pathway and the production requirements. Different fermentation conditions investigated include whole culture aeration step after anaerobic fermentation and addition of DMB and  $\delta$ -aminolevulinic acid (ALA) as supplements. *Propionibacterium freudenreichii*, the commercial producer for vitamin B12 was used as standard bacteria to compare.

## Materials and methods

**Chemicals:** Adenosylcobalamin  $\delta$ -aminolevulinic acid (ALA), Cyanocobalamin, 5, 6-dimethylbenzimidazole (DMB) and other chemicals were purchased from Sigma. All other chemicals were of reagent grade.

**Microorganisms and media:** *Lactobacillus reuteri* CICC 6226 and CICC 6130 strains were obtained from China Industrial microbiology culture collection center (CICC) and precultured in sterile de Man, Rogosa and Sharpe (MRS) medium (Oxoid, Basingstoke, UK). The modified MRS medium consisted of 30 g glucose, 5 g glycerol, 10 g peptone, 5 g yeast extract, 1 g tween-80, ammonium citrate, 5 g sodium acetate, 0.1 g  $MgSO_4 \cdot 7H_2O$ ,  $MnSO_4 (H_2O)$  and 3 g  $CaCO_3$  with 2 g  $K_2HPO_4$ ,  $CoCl_2 \cdot 6H_2O$  (100 mg/L), ALA (100 mg/L) and DMB (100 mg/L) at pH 6.5.

**Inoculum preparation and fermentation:** Both *L. reuteri* strains were incubated statically for 16 h at 37°C and 2% of the growth culture was inoculated into eight anaerobic flasks containing 100 mL of sterile modified MRS medium.

Table 1. Different growth conditions studied for *L. reuteri*.

Symbol	Growth conditions
A	DMB (100 mg/L) and aeration stage
B	DMB (100 mg/L)
C	Aeration stage
D	Without all
E	DMB (100 mg/L), ALA (100 mg/L) and aeration stage
F	DMB (100 mg/L), ALA (100 mg/L)
G	ALA (100 mg/L) and aeration stage
H	ALA (100 mg/L)

Each flask at different growth conditions is listed in Table 1. (A) Addition of DMB (100 mg/L) without ALA in which fermentation was divided into two stages. In the first, 24 h of fermentation, the bacteria were grown anaerobically at 37°C in anaerobic incubator and after that, the cultivation was shifted to the aerobic cultivation with a rotary shaker at 120 rpm for 8 h, (B) Addition of DMB (100 mg/L) without ALA and without aeration in the final fermentation process, (C) Without addition of DBM or ALA in which fermentation was divided into two stages including aeration at the end of fermentation, (D) Without DBM or ALA and without aeration in the final fermentation process, (E) With DMB (100 mg/L), ALA (100 mg/L) and aeration stage, (F) With DMB (100 mg/L), ALA (100 mg/L) and without aeration stage, (G) Without DMB (100 mg/L) and with ALA (100 mg/L) and aeration stage and (H) Without DMB (100 mg/L), with ALA (100 mg/L) and without aeration stage.

**Preparation of cell extracts:** In order to analyze vitamin B12 obtained from *Lactobacillus reuteri*, the bacterial cells were harvested by centrifugation (6,000xg, 10 min, 4°C) and washed twice with deionized water. The bacterial cell pellet was resuspended into 1 g/1 mL lysozyme buffer, incubated at 37°C for 30 min and followed by ultrasonic cell disruption for five times at 1-min interval under ice. The cell extraction was made up to a final volume of 20 mL with sodium nitrite and potassium cyanide (0.5: 0.2 g/100 mL) and then autoclaved at 120°C for 15 min. The mixture was cleared by centrifugation (8,000xg; 10 min) and the supernatant was passed through the solid-phase extraction (SPE) column (500 mg C18 end-capped column with a 3 mL reservoir volume) previously activated with 2 mL of acetonitrile. The column was washed twice with distilled water to remove salts and other hydrophilic contaminants. Subsequently, the sample was eluted with 1 volume of 50% acetonitrile and concentrated to dry in vacuum at 30°C. The residue was dissolved in 1 mL of sterile distilled water and stored in the dark at 20°C until further use.

**HPLC analysis:** The corrinoids from *L. reuteri* cell-extracts were purified and analyzed using Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA) that comprised of an auto sampler and an Ultimate 3000 Variable Wavelength detector (UV) set at 361 nm. Vitamin B12 concentration was analyzed at a flow rate of 0.7 mL/min.

The system was completed with an Amethyst column type Acclaim C18-H (5  $\mu$ m, 4.6x250 mm). Two mobile phases (A, B) were used in order to purify the corrinoid. The composition was methanol: distilled water (60:40) and (10:90) respectively with added 0.07 mL phosphoric acid. The samples and standards were dissolved in the mobile phase and 10  $\mu$ L sample volume was injected into the column at 30°C.

**Mass spectrometry:** Mass spectrometric analysis of *L. reuteri* cell extraction was performed by the liquid chromatography/mass spectrometry-ion trap-time of flight (LC-MS-IT-TOF) (Shimadzu, Kyoto, Japan). Analysis parameters: Electrospray ion source; Interface voltage of 3.50 kV, Nebulizer gas flow of 1.5 L min<sup>-1</sup>, CDL temperature of 200°C, Block heater temperature of 200°C, Detector voltage of 1.60 kV, Drying gas of 100 kpa, Ion accumulation time of 10 msec, MS range of m/z 300 to 1500, CID parameters as energy of 50%, collision gas of 100%, repeated of 10. MS data were processed with LC-MS solution ver. 3.4 software (Shimadzu). Ten microliters of the sample solution was introduced into the ESI ion source of the mass spectrometer via the Autosampler of HPLC system with C18 column (150x2.0 mm; Shimadzu, Kyoto, Japan).

## Results and discussion

**HPLC results:** UV-Vis spectra data and retention times obtained by HPLC analysis showed that both *L. reuteri* strains showed the peaks with retention times nearly agreed with that of the cyanocobalamin standard under the conditions of (A) with DBM, ALA and two stages including aeration at the end of the fermentation. As shown in Fig. 1, the cell-extracts from *L. reuteri* CICC 6226 under conditions (A) revealed a peak with a retention time of 14.287 min (Fig. 1A) which was agreed with the retention time of 14.280 min for cyanocobalamin standard (Fig. 1B) and with the cell-extracts from *P. freudenreichii* (Fig. 1C). By contrary, all other samples of B, C, D, E, G and H did not show any peak with a retention time agreed with the cyanocobalamin standard. The cell extraction of both strains under all of the conditions as well as *P. freudenreichii* showed the same peak at about 12.87 min which was approx. 1.4 min before the peak of cyanocobalamin standard in Fig. 2.

**Mass spectrometry results:** The mass spectra (Fig. 3) of the corrinoid produced by *L. reuteri* CICC 6226 and CICC 6130 under conditions (A) 1355.559 (Fig. 3A) was identical to the complete molecule of the active form of vitamin B12 cyanocobalamin standard 1355.557 (Fig. 3B) as well as the mass spectra of the *P. freudenreichii*. *Lactobacillus reuteri* recently has been studied as one of the anaerobic vitamin B12 producers because of its capability to utilize glycerol and excrete reuterin through a cobalamin-dependent glycerol dehydratase without exogenous cobalamin in a vitamin B12-free medium (Taranto *et al.*, 2003).

Fig. 1. (A) RP-HPLC chromatogram of the cell extraction from *L. reuteri* and UV-Vis spectrum of the peak with a retention time of 14.287 min, (B) For reference, chromatogram obtained with the standard of cyanocobalamin 14.280 min and (C) For chromatogram of the cell extraction from the standard bacteria, *P. freudenreichii* with a retention time of 14.287 min.

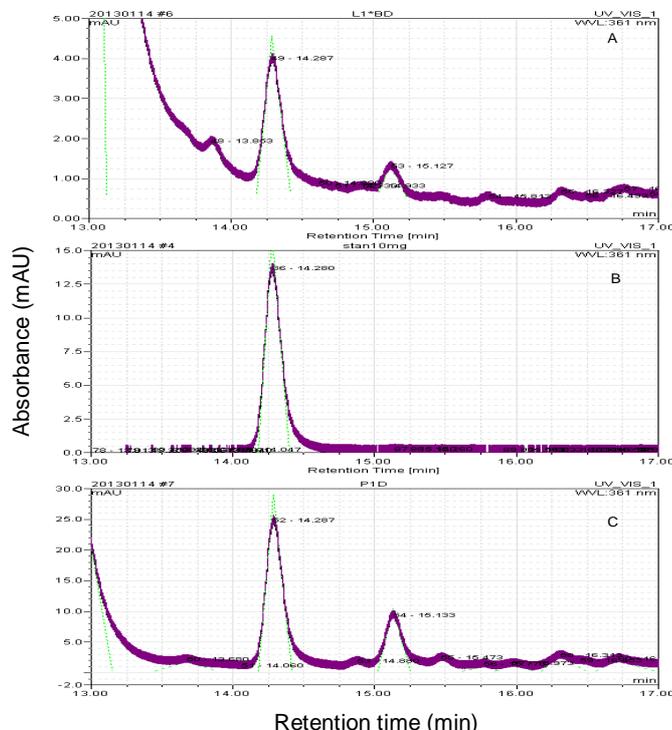


Fig. 2. (A) RP-HPLC chromatogram of the cell extraction from *L. reuteri* with addition of ALA and the UV-Vis spectrum of the peak with a retention time of 12.813 min. (B) Cell extraction of *L. reuteri* without addition of ALA revealed small peak with a retention time of 12.907 min. (C) Cell extraction of *P. freudenreichii* showed peak with a retention time of 12.860 min.

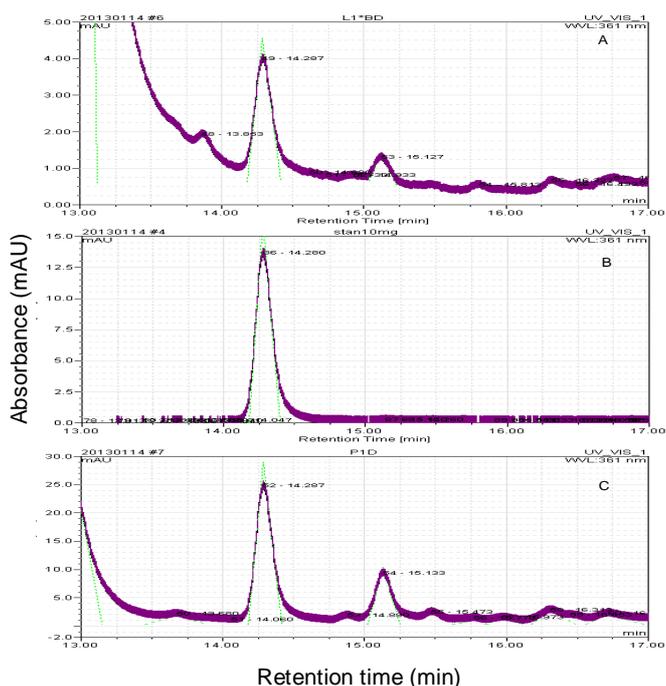
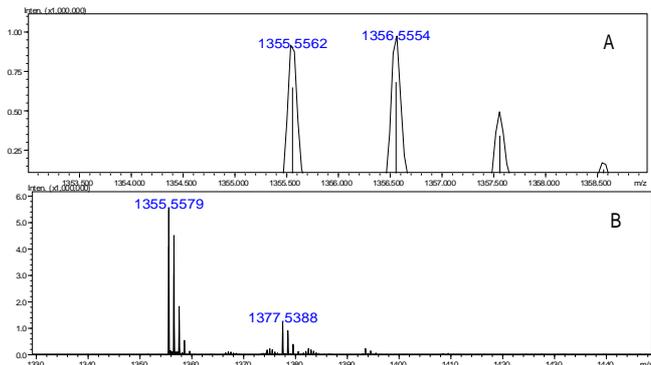


Fig. 3. (A) Mass spectrometry spectrum of the corrinoids purified from *L. reuteri*. (B) Mass spectrometry spectrum of the cyanocobalamin standard.



Moreover, the necessary genes for the anaerobic vitamin B12 biosynthesis pathway have been identified in the chromosome of *L. reuteri* (Santos *et al.*, 2008). However, it has proven after few years studies that pseudovitamin-B12 is the dominant corrinoid produced by *L. reuteri* instead of vitamin B12 on account of the absence of genes involved in the synthesis of DMB in *L. reuteri* DNA (Santos *et al.*, 2007). DMB plays a key role in the completion of synthesis vitamin B12. Therefore, in our study, different fermentation conditions have been investigated with the aim of inducing the bacteria to produce the active form of vitamin B12. This approach led to fruitful results. From the HPLC and mass spectrometry data, the wild type *L. reuteri* strains have been demonstrated to produce  $\alpha$ -(5,6-dimethylbenzimidazolyl)-cobamidcyanide (cyanocobalamin) under certain conditions with the addition of DMB and ALA as well as the aeration stage at the end fermentation stage (A). We successfully stimulated *L. reuteri* CICC 6226 and CICC 6130 strains to produce 44.59  $\mu$ g and 6.83  $\mu$ g cyanocobalamin per L culture respectively through two different approaches.

The first approach was to provide the critical precursor,  $\delta$ -aminolevulinic acid (ALA) in the beginning of the biosynthesis pathway of vitamin B12 to enhance the production toward cobinamide prior to the final step of synthesis vitamin B12. Numerous studies have been focused on the overexpression of some genes (hemA, hemL) to increase the productivity of ALA for optimizing the production of vitamin B12 as a final product (Kiatpapan and Murooka, 2001; Biedendieck *et al.*, 2010). As for the second approach, the final step of the biosynthesis of vitamin B12 (cobinamide bioconversion) in *L. reuteri* was stimulated to function toward cobalamin with lower (Co $\alpha$ ) ligand base which is 5,6-dimethylbenzimidazole (DMB) instead of pseudovitamin B12 with adenine as the lower (Co $\alpha$ ) ligand by adding DMB (100 mg/L) directly into the culture medium under aerobic conditions in the last phase of the fermentation. The addition of DMB without aeration was not enough to insert exogenous DMB into the lower ligand producing cobalamin.

It is evident that this process and the sequent reactions to produce cobalamin also require molecular oxygen. Furthermore, the gentle aeration of the culture at the end of the fermentation for 8 h appears to be no substantial effects on the growth of *L. reuteri* and the biomass. On the other hand, when one compares the UV spectra of the cell extraction of *L. reuteri* and with cyanocobalamin standard, the cell extraction of the both strains under the same conditions showed the same pattern and spectra with retention time about 12.890 min with that of *P. freudenreichii*. The difference in the retention time between this peak and cyanocobalamin standard was about 1.4 min. These peaks have shown different peak areas after the addition of ALA and the peak areas were increased after the addition of ALA as a supplement in the culture medium. We have deduced from our HPLC data and the previous studies (Taranto *et al.*, 2003; Santos *et al.*, 2007) that the peaks with retention time about 12.890 min were pseudovitamin B12 and the difference in retention times between the samples and the cyanocobalamin standard due to the difference in the lower ligand, where the DMB moiety was replaced by adenine. We have also noticed this similar behavior with *Bacillus megaterium*, another producer for vitamin B12 in our separate experiments (data not shown). It is clear that pseudovitamin-B12 is the most common corrinoid in all of the anaerobic producers of vitamin B12. Therefore, the key issue for the production of the active form of vitamin B12 in these bacteria is the conversion of cobinamide to cobalamin through the replacement of the GDP group of adenosyl-GDP-cobinamide with  $\alpha$ -ribazole catalyzed by CobS (anaerobic pathway). This nucleotide loop consists of the DMB attached to ribose. The synthesis of DMB is derived from riboflavin by the action of BluB in the presence of molecular oxygen (Escalante-Semerena, 2007; Scott and Roessner, 2007).

In *P. freudenreichii*, the BluB protein is fused to the CobT enzyme (BluB-CobT) to synthesis what is known as  $\alpha$ -ribazole-P which linked to the complete corrinoid (cobamide) to reveal cobalamin (Falentin *et al.*, 2010), but *L. reuteri* has no homologue of bluB and unable to synthesis DMB (Santos *et al.*, 2008). Thus, it needs exogenous DMB in *L. reuteri*. From the HPLC data we also noticed that *L. reuteri* could be a perfect platform for the synthesis of vitamin B12. The peak area of the pseudo-B12 indicate that the concentration of cobinamide was incredibly high when adding the ALA in the culture medium due to the lack of the branched pathway of HemE in *L. reuteri*. This means that most of ALA and uroporphyrinogen III (vitamin B12 precursor) flowed towards vitamin B12 pathway unlike *P. freudenreichii* and *B. megaterium* in which a large part of ALA flowed towards hemE along the branched pathway (Biedendieck *et al.*, 2010). However, the problem of *L. reuteri* remains in the synthesis of DMB and the formation of active form of vitamin B12.

Therefore, in this study, the key issues of this biosynthesis have been clarified. In addition, we initiated to deal with these issues and we succeeded to produce an active vitamin B12 [DMB] (cyanocobalamin) for the first time in the scientific world. We also carried out to overexpress mutant hemA and hemL form *Rhodobacter sphaeroides* 2.4.1 to increase ALA yield and cobA and bluB-cobT from *P. freudenreichii* CICC 10019. Our aim was to increase the flow of uroporphyrinogen III toward the rest of the vitamin B12 pathway in addition to synthesis DMB and incorporate it into cobamide and complete the synthesis of vitamin B12, into *L. reuteri* under control of the nisA promoter using pNZ8148 plasmid. Unfortunately, our strategies have not been successful so far. This might be because *L. reuteri* does not have the regulatory genes integrated into the chromosome. In this case, it is supposed to use two plasmids including the regulatory plasmid pNZ9530 (nisRnisK) along with the expression plasmid (Eichenbaum, 1998). Recently, there was a great deal of interest on the probiotic effect of *L. reuteri* in addition to its applications in food industries. Demonstration of *L. reuteri* to produce an active vitamin B12 could raise its value extremely more for the production of fermented foods and feed with increased vitamin B12 contents.

## Conclusion

In the present study, we have demonstrated the ability of two wild type strains of *L. reuteri* to produce  $\alpha$ -(5,6-dimethylbenzimidazolyl) cobamidcyanide or cyanocobalamin the active form of vitamin B12 by providing the required supplements (ALA and DMB) for the synthesis of vitamin B12, under the appropriate conditions to complete the formation of vitamin B12. In addition, the possibility of *L. reuteri* to be a good candidate for the production of vitamin B12 has been discussed for further studies.

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