Enhancing vitamin $\text{B}_{12}$ content in co-fermented soy-milk via a Lotka Volterra model

Abstract: Soybean products are popular because of its taste, digestibility, and health benefits. However, soybean lacks vitamin, mainly the low water-soluble vitamin $\text{B}_{12}$. This study investigated the effects of fermentation conditions on the synthesis of vitamin $\text{B}_{12}$, production of metabolites, and growth of *Lactobacillus reuteri* and *Propionibacterium shermanii* in fermented soy-milk. A Lotka Volterra model was successfully employed to describe the competition relationship between the two microorganisms under various fermentation conditions. A quadratic function between the ratio of interaction coefficients and vitamin $\text{B}_{12}$ content was found. Higher vitamin $\text{B}_{12}$ in soy-milk can be produced when the ratio of interaction coefficients approach to one. Compared with other fermented soybean products, fermented soy-milk contains more acetate, ethanol, and propionic acid. This study successfully demonstrated a mathematical model to enhance soy-milk vitamin $\text{B}_{12}$ production.

Keywords: *Lactobacillus reuteri*; *Propionibacterium shermanii*; co-fermentation; modeling; vitamin $\text{B}_{12}$.

Introduction

Soybean products have been demonstrated to be a good substitution of meat protein due to its similar taste, high digestibility and health benefits [1, 2]. Traditional tofu, as a typical oriental soybean product, is made through a serial process of soaking, grinding, heating, fiber removal, coagulation, and pressing [3]. Furu and stinky tofu are fermented tofu products with brine by mold and bacteria [4]. Compared to soy-milk, these fermented products have no or less characteristic beany flavor and the bitter or astringent taste [5]. They are normally inexpensive and highly nutritious, therefore, studies had demonstrated them to be substitutions of meat; when cooked together with vegetables or in soups as a high quality protein supplementation, they are also suitable for the seniors and vegetarians [3]. However, sufu and stinky tofu have high concentration of salt and ammonia [3, 6], which is not acceptable for
many customers. A high sodium daily-intake might cause a long-term risk of cardiovascular issues [7]. Soy-milk, on the other hand, does not have high salt and ammonia, but it contains a low vitamin content, especially the water-soluble vitamin B$_{12}$ [8]. Vitamin B$_{12}$ (cobalamin) works as a cofactor involved in a variety of enzymatic reactions [9]. Vitamin B$_{12}$ deficiency might lead to the disturbance in cell division, neuropathy, nervous system disease, and pernicious anemia [10]. To prevent such fatal deficiency diseases, 2.4 μg of vitamin B$_{12}$ daily intake is suggested [11]. Vitamin B$_{12}$ is exclusively synthesized by certain bacteria and archaea, and is accumulated in predators’ bodies in the food chain [12].

A co-fermentation with Propionibacterium shermanii and Lactobacillus reuteri was employed to solve the problem of low cobalamin concentrations in fermented soy-milk. Propionibacterium shermanii with an ability of high cobalamin production prefers to consume lactate as the main energy and carbon source [13, 14]. This can reduce the stress of lactate on L. reuteri and retard the decrease of pH. Moreover, Propionibacterium spp. has a 100-fold stronger activity of hydrolyzing triglycerides of fat, compared with lactic acid bacteria [15]. Hence glycerol produced by P. shermanii induces vitamin B$_{12}$ dependent enzyme in L. reuteri. The synthesis of DMBI 5,6-dimethylbenzimidazole, an important precursor of cobalamin, can only be formed in the presence of oxygen by Propionibacterium freudenreichii and P. shermanii [16]. Because of this, after several days of fermentation under anaerobic conditions with P. freudenreichii or P. shermanii, the fermentation should be switched to aerobic conditions. However, Santos et al. [17] found that L. reuteri has the ability to form DMBI without oxygen. In previous work, vitamin B$_{12}$ production from co-fermentation was obviously higher than those from L. reuteri or P. shermanii single fermentations [18], indicating a big influence of fermentation type on the interaction of bacteria and synthesis of vitamin B$_{12}$. A Lotka Volterra model of competition, historically proposed in ecology as a mechanistic model, was introduced into this work to interpret the interactions between two microorganisms under different conditions. Coefficients were used to explain the underling mechanism. This model has been introduced into several experiments such as the growth of LAB, coliforms, pseudomonads, Brochothrix, Salmonella, and yeasts on sliced pork shoulder; growth of Aeromonas hydrophila on fish; and interactions of yeast to yeast and yeast to bacterium during the ripening of cheeses [19]. As to our knowledge, this model can be applied to provide guidance and evaluate results of co-fermentation.

Materials and methods

Microorganisms and culture conditions

Lactobacillus reuteri ZJ03 and P. shermanii ZJ01 were taken from the culture collection of Key Laboratory for Food Safety of Zhejiang Province. The stock of cells was maintained in glycerol 50% (v/v) at −70°C. The bacteria were propagated in vitamin B$_{12}$ test broth (Luqiao, Beijing, China) in standing cultures overnight at 37°C.

One milliliter of fermented soy-milk was mixed with 9 mL of saline solution. The sample was diluted to corresponding concentrations and spread onto solid agars such as MRS (pH of 5.0) and NaLa agar [20]. MRS agar was incubated at 37°C for 72 h and NaLa agar was incubated at 30°C for 7 days. Lactobacillus reuteri ZJ03 was counted from MRS agar of the white shiny smooth colonies. Propionibacterium shermanii ZJ01 was identified and counted from NaLa agar by the morphology of 1.0–2.5 mm, dull brown, lighter margin colonies. A subtraction method, as a control, could also be used to calculate counts of Propionibacteria by subtracting the number of L. reuteri from the total count in NaLa agar.

Fermentation

A basic medium in a glass flask was prepared using 100 mL soy-milk, 5 g glucose, 1 mL of 10$^8$ CFU/mL of Lactobacillus reuteri inoculum and 1 mL of 10$^8$ CFU of P. shermanii inoculum. The medium were adjusted pH to 6.5 and conducted at 30°C, if it was not mentioned separately.

To study the effect of pH on fermentations, initial pH values were adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0 for this set of experiments. To study the effect of temperature on fermentations, experiments were conducted under 28, 30, 35, and 37°C. To study the combination effects of aerobic/anaerobic fermentation, experiments were conducted respectively under 6-day anaerobic and 1-day aerobic, 5-day anaerobic and 2-day aerobic, and 4-day anaerobic and 3-day aerobic fermentation conditions, respectively. All other trials, unless specified, were carried out at 30°C under 5-day anaerobic followed by 2-day aerobic fermentation conditions.

Determination of vitamin B$_{12}$ and metabolites

Vitamin B$_{12}$ was analyzed using a microbiological assay modified by Denter [21] and HPLC method [22]. Vitamin B$_{12}$ was extracted from 1 mL of soy-milk with 10 mL sodium acetate buffer (pH 6.0) in present of KCN and heated in a
water bath for 30 min at 70°C. Then, the pH of the solution was adjusted to 7.0 and mixed with 10 mL hexane (Extra pure N-hexane, Merck, Darmstadt, Germany) then centrifuged for 15 min at 4010 g (Varifuge 3.0, Heraeus Instruments, Hanau, Germany). The acesphous phase was collected and passed through a solid phase extraction column (SPE) (CEC181M6 United Chemical Technologies, Bristol, PA, USA), which had been washed by 3 mL methanol (Merck, Darmstadt, Germany) and 3 mL double distilled water (DDW) [from Reversed osmosis Mill-Q water (18 Q) (Millipore, Billerica, MA, USA)], with the aid of a pump (AL 15, Knf Neuberger, Hamburg, Germany) to control the speed of drops at one drop per second. After 3 times washing by ultra purified water, 3 mL methanol was used as the eluate. After the solvent was evaporated to dry, the residue was dissolved by 1 mL ultra purified water. The solvent was filtered through a membrane filter (0.2 μm) (Macherey-Nagel, Düren, Germany) and the filtrate was analyzed by HPLC (Waters E2695, USA) using a RP-18 column (250*4 mm I.D., 5 μm, Merck, Darmstadt, Germany).

All chromatographic separations were carried out at room temperature. A flow of 0.5 mL/min, methanol with 0.1% formic acid (A) (Merck, Darmstadt, Germany) and ultra purified water with 0.1% formic acid (B), which were degassed by an ultrasonic water bath (Sonorex TK 52, ultrasonic waterbath, Bandelin electronics, Berlin, Germany), were used as mobile phases and the gradient elution was programmed as follows; 0–2 min 20% A; 2–3 min 20–25% A; 3–11 min 25–35% A; 11–19 min 35–20% A; 20–22 min 100–100% A; 22–26 min 100–20% A; 26–36 min 20% A. The injection volume was 100 μL and the column eluate was monitored by Diode Array Detector (Waters, USA) at 361 nm wavelength.

Metabolites were detected using HPLC (Waters E2695, USA) with an organic acid column (850 BP-OA H+, 300*7.8 mm, Benson Polymeric, Sparks, USA) as a solid phase. All chromatographic separations were carried out at 60°C. A flow of 0.6 mL/min with 26 mM sulfuric acid was used as the mobile phase. The injection volume was 10 μL and the column eluate was monitored by Lachrom RI Detector. One milliliter fermentation liquid was centrifuged for 10 min at 17,000 g (Biofuge pico centrifuge, Heraeus Instruments, Hanau, Germany). Ten microliter supernatant was injected into HPLC for analysis.

Lotka Volterra model development/ implementation

\[
\frac{dL}{dt} = \mu_{\text{max}} L \left( \frac{Q_1}{1 + Q_1} \right) \left( 1 - \frac{L - aP}{L_{\text{max}}} \right) \quad (1)
\]

where \(Q_1\) stands for the maximum species growth rate, \(L_{\text{max}}\) is the maximum CFU during fermentation. The coefficients of \(a\) and \(b\) means the interspecific competition parameters of \(P\). shermanii on \(L\). reuteri, vice versa.

An assumption was made that both microorganisms grew naturally without any inhibition from themselves. \(L\) and \(P\) stand for CFU of \(L\). reuteri and \(P\). shermanii at time \(t\). \(Q_1\) and \(Q_2\) respectively represent the physiological state of both microorganisms. \(\mu_{\text{max}}\) and \(\mu_{\text{max}}^P\) separately show the maximum species growth rate. \(L_{\text{max}}\) and \(P_{\text{max}}\) stand for the maximum CFU during fermentation. The coefficients of \(a\) and \(b\) means the interspecific competition parameters of \(P\). shermanii on \(L\). reuteri, vice versa.

According to the assumption made, \(Q_1/(1 + Q_1)\) was set as 1. The integration of equation was made from \(t_{i-1}\) to \(t_i\). The differential equations (Eq. 1, Eq. 2) can not be solved manually. Hence, least squares method was introduced to estimate the coefficients of \(a\) and \(b\) using Matlab (Version 5.3.0.10183, Mathworks Inc., Natick, MA, USA). The transpose of \(A\) is \(A^T\).

\[
\ln L_{i-1} - \ln L_{i-1} = \mu_{\text{max}}^L (t - t_{i-1}) - \mu_{\text{max}}^L A_{i-1} L_{\text{max}}^L - \mu_{\text{max}}^a A_{i-1} L_{\text{max}}^a A_{2i} \quad (3)
\]

\[
\ln P_{i-1} - \ln P_{i-1} = \mu_{\text{max}}^P (t - t_{i-1}) - \mu_{\text{max}}^P A_{i-1} L_{\text{max}}^P - \mu_{\text{max}}^b A_{i-1} L_{\text{max}}^b A_{2i} \quad (4)
\]

\[
A_{ij} = \int_{t_{i-1}}^{t_i} L(t) \, dt \\
= 1, 2, 3...m
\]

\[
AX = B_L \quad (3-6)
\]

\[
AX = B_P \quad (3-7)
\]

\[
A = \begin{bmatrix}
-t_1 - t_0 & -A_{11} & -A_{21} \\
\vdots & \vdots & \vdots \\
-t_m - t_{m-1} & -A_{1m} & -A_{2m}
\end{bmatrix}
\]

\[
X = \begin{bmatrix}
\mu_{\text{max}}^L \\
\mu_{\text{max}}^P \\
\mu_{\text{max}}^a \\
\mu_{\text{max}}^b \\
L_{\text{max}}^L \\
L_{\text{max}}^P
\end{bmatrix}
\]

\[
Y = \begin{bmatrix}
\mu_{\text{max}}^P \\
\mu_{\text{max}}^P \\
\mu_{\text{max}}^a \\
\mu_{\text{max}}^b \\
L_{\text{max}}^P \\
L_{\text{max}}^L
\end{bmatrix}
\]
Results and discussions

Temperature effects on co-fermentation

Temperature displayed a mild influence on interactions between these bacteria. With the increase of temperature, the inhibition effects of both bacteria increased (Table 1). In this study (Figure 1), 30°C is an optimal temperature for co-fermentation to produce vitamin B_{12}. An unexpected drop in cell numbers for *L. reuteri* and *P. shermanii* was observed from 28 to 37°C (Figure 2). The decrease of final concentrations of ethanol (Table 2) and increase of final concentrations of propionic acid (Table 2) from 28 to 37°C can be recognized as a reason for low cobalamin production and low cell densities of *L. reuteri*, confirmed by increasing values of interaction coefficient a (Table 1). Our data suggested that dramatic changes of metabolism were observed at 37°C. It is may due to the suitable temperature for *L. reuteri* to produce more lactate. Meanwhile, *P. shermanii* prefers to utilize lactate as primary carbon resources, leading to increasing of propionic acid. There is no report about the relationship between temperature and cobalamin production before. But some researchers [14, 23–25] reported a correlation between temperature and 3-HPA production or propionic acid, in which a cobalamin dependent enzyme was involved. Doleyres et al. [23] reported no significant difference in 3-HPA production at temperatures between 15 and 37°C. Another contradictory result suggested that 3-HPA production at 37°C was significantly higher than at other temperatures in any kind of media [25]. The optimal growth temperature for *Propionibacterium* spp. is however, almost 30°C [24].

### Initial pH effects on co-fermentation

With the increase of initial pH values, both bacteria had a synergistic effect on each other. But these phenomena did not enhance the production of vitamin B_{12}. pH is essentially important in influencing metabolites and cobalamin production. Both microorganisms have their own optimal pH and the corresponding suitable ranges. The optimal pH for 3-HPA production is 6.0 [24], whereas the best pH for propionic acid is between 7.0 and 7.2 [14]. The question of the optimal initial pH for cobalamin production in co-fermentation was solved by our work (Figure 1). At pH 6.5–7.0 the highest values of cobalamin were reached. Interestingly, as is shown in Table 1, the ratio of a/b at pH 6.5 and pH 7.0 were 13.54/16.64 and −10.19/−10.31, respectively. Except for final concentrations of *L. reuteri* at pH 6.5, no huge difference was observed between both microorganisms content among various pH conditions (Figure 2). At pH 6.5 they had an inhibitory effect, but at pH 7.0 they changed to a synergistic effect. Moreover, a very strong influence on production of ethanol, propionic acid, and acetate was observed (Table 2). A possible interpretation of high cobalamin production is that a higher production of propionic acid needs more cobalamin under acidic condition (pH 6.5–7.0), as reported by Hsu and Yang [26]. Another explanation could be the low activity of 3-HPA generation above pH 7.0 [24], whereas more ethanol was generated to balance the redox reaction in *L. reuteri*.

### Oxygen supplementation effects on co-fermentation

Oxygen, as mentioned above, is involved in the DMBI generation in *P. freudenreichii*. In the presence of oxygen, growth is slower due to the inhibition of propionic acid, acetate, and succinate formation, but pyruvate is
accumulated [27]. However, propionic acid, an inhibitory factor of both microorganisms, can be decomposed in the presence of oxygen. Some researchers [28, 29] have conducted an oxygen cycle to improve cobalamin production by mediating catabolism of glucose to propionic acid and acetate in the presence and absence of oxygen [14].
Oxygen also affects *L. reuteri* to synthesize more heme against toxic forms of oxygen [30]. In this study (Figure 1), 2-day aerobic fermentation showed a higher productivity of cobalamin than 1-day or 3-day aerobic fermentation. But an obvious increase inhibitory effect of bacteria can be observed from an increase of interaction coefficients during the increase of aerobic fermentation duration. The CFU of *L. reuteri* increased during the increase of oxygen supplied days (Figure 2). *Propionibacterium shermanii* demonstrated the opposite trend (Figure 2). Some researchers [29, 31] found that low dissolved oxygen was advantageous for cell growth, propionate decomposition and acetate production by *P. shermanii* decrement, which was also confirmed in this study (Table 2). The dissolved oxygen obviously led to a reduction of final ethanol concentrations (Table 2).

**Interaction and co-fermentation**

A Lotka Volterra model, known as an ecological predator-prey model, was applied to describe the competition between microorganisms. The interaction coefficients, which describe the antagonistic activities, were obtained by fitting the Lotka Volterra model with least square methods. The coefficients of a and b can be interpreted as the interspecific competition parameters of *P. shermanii* on *L. reuteri*, and vice versa. The average values of interaction coefficients of a (−4.66) and b (−3.60) from this study represented a less negative effect from *P. shermanii* on *L. reuteri* and a negative effect from *L. reuteri* on *P. shermanii*. However, some researchers [24] reported that the maximal cell numbers of *L. acidophilus* and *P. shermanii* were higher than in single culture fermentation.

With the exception of pH 6.5, all fermentations with high productions of cobalamin did not show strong antagonistic effects between the two microorganisms (Table 1). Experiments of oxygen supply for 1 day acquired huge negative values of interaction coefficients. It can be explained that oxygen to some extent became the main inhibitor for the growth of both microorganisms. Interaction coefficients were changing from positive values to negative values during fermentations with an increase of initial pH from 6.0 to 8.0. This means a high initial pH value is beneficial for growth of both bacteria. No significant difference of interactions was found in fermentation under different temperatures.

A synergistic effect in the co-fermentation of *L. acidophilus* and *P. shermanii* was described by Liu and Moon [24]. They reported no lactate accumulation in the medium. Acetic acid production rates per generation were lower in mixed cultures and growth rate was faster than before. Results from this study agreed with previous results [24] and partly confirmed them as well. The robust growth of mixed cultures was also indicated in this study. Fast increase of propionic acid, ethanol production, and OD values were observed (data not shown), along with an accumulation of lactate observed, particularly in fermentation at 37°C (Table 2). *Propionibacterium* sp. can reduce the lactate stress on *L. reuteri* and retard the decrease of pH. Moreover, at the same time, *L. reuteri* can decompose proteins from soymilk relying on full proteolytic system to meet the nitrogen requirement of itself and *P. freudenreichii* with a low ability of protease. Another hypothetical assumption is about the synthesis of Dmbi. 5,6-dimethylbenzimidazole, an important precursor of cobalamin, can only be formed in the presence of oxygen by *P. freudenreichii* or *P. shermanii* [16]. Because of this, after several days of fermentation under anaerobic conditions with *P. freudenreichii* or *P. shermanii*, the fermentation should be switched to aerobic conditions. However, Santos et al. [17] found that the gene of cobT of *L. reuteri* is 59% similar with *Salmonella typhimurium*, which could mean that *L. reuteri* has the ability to form Dmbi without oxygen. Furthermore, some analogs can improve production of cobalamin by protecting an inhibitory riboswitch [32].

Some researchers demonstrated that spent media cultured with LAB strains led to a low cell concentration of *P. shermanii*, which produced more cobalamin than before [33]. Nevertheless, mixed cultures in this study produced 1.6–2.4 fold more cobalamin than single fermentation (data not shown). For further work, a doubtless conclusion can be made that a co-fermentation with *P. shermanii* and *L. reuteri* can lead to a high production of cobalamin in soymilk. Correlations between the ratio of a/b and vitamin B₁₂ production were also found. According to Figure 3, an obvious conclusion can be made that more vitamin B₁₂ production (Figure 1) can be produced.
when values of $a/b$ approaches to 1. This means that both bacteria contribute to vitamin $B_{12}$ production. The model is therefore demonstrated its feasibility in describing the response of vitamin $B_{12}$ production and predicting a response value within the appropriate ranges.

**Conclusions**

A Lotka Volterra model was successfully employed to describe the competition relationship between $L. reuteri$ and $P. freudenreichii$ microorganisms under various fermentation conditions of anaerobic/aerobic, initial pH, and temperature. With the increase of aerobic fermentation days and the rising of fermentation temperature, the inhibition effects between microorganisms were rising. During the increase of initial pH values, the synergistic effects were developing. A quadratic function was set up to predict vitamin $B_{12}$ by values of $a/b$. This model has achieved to explain interactions of two bacteria and provide a useful approach to further increase yield of vitamin $B_{12}$, even other relative fermented products.

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