植物由来の乳酸菌を用いたビタミンB12強化ザワークラウト
(キャベツの漬物)の調製

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Determination of Vitamin B₁₂ Content of Sauerkraut (Pickled Cabbage) Products and Plant-Derived Lactic Acid Bacteria

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Vitamin B₁₂ content of four types of commercially available sauerkraut (pickled cabbage) products was determined and characterized. Although one product contained considerable vitamin B₁₂ content (approximately 0.5μg/100g wet weight), the remaining three products contained only traces of vitamin B₁₂. Subsequent liquid chromatography/electrospray ionization-tandem mass spectrometry analyses revealed that most of the corrinoids found in the high-vitamin B₁₂ sample was pseudo-vitamin B₁₂, an inactive corrinoid compound, thereby suggesting that this sauerkraut product is not a suitable source of vitamin B₁₂. Vitamin B₁₂ content in 16 Lactobacillus strains isolated from vegetables and fruits and their products was determined. Although all lactic acid bacteria grew well in vitamin B₁₂-deficient medium, only eight L. plantarum strains grew well in cabbage-extract medium, with L. plantarum FSCM 2-12 producing the highest biomass and vitamin B₁₂ content.

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Key words: corrinoids, Lactobacillus plantarum, pickled cabbage, sauerkraut starter, vitamin B₁₂

Vitamin B₁₂ (or cobalamin), an essential nutrient for humans, is unique because it is only synthesized by certain bacteria⁶. In nature, vitamin B₁₂ synthesized by bacteria is taken up by plankton, which serve as a crucial food source for fish, shellfish, and other large aquatic organisms⁵. Vitamin B₁₂ becomes concentrated in the bodies of higher predators in the food chain, and is eventually ingested by humans⁵. It is the only vitamin that is absent in plant-derived foods because plants cannot synthesize vitamin B₁₂⁶. People who do not consume animal-derived foods, such as vegetarians, are at a high risk of developing vitamin B₁₂ deficiency, which has the major symptoms of neuropathy and megaloblastic anemia⁶. Therefore, the identification of plant-derived foods containing high levels of vitamin B₁₂ is necessary to prevent vegetarians from developing vitamin B₁₂ deficiency.

Pickled vegetables, such as sauerkraut, are usually produced by spontaneous fermentation involving lactic acid bacteria⁷. Globally, sauerkraut is the most common cabbage-containing product and is commonly used in vegetarian dishes⁸. The vitamin B₁₂-synthesizing lactic acid bacteria Lactobacillus coryniformis and L. plantarum have been isolated from Japanese pickled vegetables⁸. Sauerkraut products containing considerable levels of vitamin B₁₂ could be excellent sources of vitamin B₁₂ for vegetarians.

In the present study, we analyzed the vitamin B₁₂ content of four sauerkraut products consumed by European vegetarians. In addition, 16 strains of

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lactic acid bacteria isolated from plants (vegetables and fruits) and their products were characterized and used as starters to produce vitamin B₁₂-enriched sauerkraut.

Materials and methods

1. Materials

Sauerkraut products were purchased from markets in Japan and Germany. Various Lactobacillus strains were obtained from the NODAI culture collection center (Tokyo University of Agriculture, Tokyo, Japan) and FATU culture collection (Faculty of Agriculture, Tottori University, Tottori, Japan) as isolates from plants and vegetable products. The selected bacterial strains are listed in Table 1. Vitamin B₁₂ was obtained from Sigma (St Louis, MO, USA) and vitamin B₁₂ assay medium, based on L. delbrueckii (formerly L. leichmannii) ATCC 7830, was obtained from Nissui (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and was also used as the vitamin B₁₂-deficient Lactobacillus medium for bacterial growth experiments. Cabbage-extract medium was prepared by homogenizing 250 g of cabbage in 100 mL of distilled water, using a mixer (TML 160; Tescom & Co., Ltd., Tokyo, Japan). The homogenate was filtered with cotton cloth, and the filtrate was autoclaved and subsequently centrifuged at 8,500 g for 10 min to remove insoluble material. The supernatant fraction was used as cabbage-extract medium.

2. Extraction and assay of vitamin B₁₂ from sauerkraut products

Sauerkraut products were squeezed to remove the juice fraction and aliquots were subsequently homogenized using a mixer (TML160) to obtain samples for the vitamin B₁₂ assay. Briefly, vitamin B₁₂ compounds were extracted from 2.0-g samples of sauerkraut homogenate by boiling under acidic conditions (pH 4.5) in the presence of potassium cyanide and were subsequently assayed using a previously described microbiological method based on L. delbrueckii ATCC 7830. Because L. delbrueckii ATCC 7830 can utilize alkali-resistant factors for growth, deoxyribosides and deoxyribonucleotides as well as vitamin B₁₂ assay values were corrected by subtracting the contribution of the alkali-resistant factors. The vitamin B₁₂ assay was performed in three independent experiments.

3. Bioautography of vitamin B₁₂ compounds using vitamin B₁₂-dependent Escherichia coli 215

Bioautography of corrinoid compounds was performed as described previously. Each vitamin B₁₂ extract (50 μL), prepared as previously described, was partially purified and concentrated using a Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, USA) that had been washed with 5 mL of 75% (v/v) ethanol, equilibrated with 5 mL of distilled water, and washed with 5 mL of distilled water. Vitamin B₁₂ compounds were eluted through the cartridge using 2 mL of 75% (v/v) ethanol. The eluate was evaporated in a centrifugal concentrator (Integrated

<table>
<thead>
<tr>
<th>No.</th>
<th>Strains</th>
<th>Collection name</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td><em>L. coryniformis</em> subsp. torquens 1051T</td>
<td>NODAI</td>
</tr>
<tr>
<td>2</td>
<td><em>L. coryniformis</em> subsp. coryniformis 1638T</td>
<td>NODAI</td>
</tr>
<tr>
<td>3</td>
<td><em>L. plantarum</em> subsp. argentoratensis 0653T</td>
<td>NODAI</td>
</tr>
<tr>
<td>4</td>
<td><em>L. plantarum</em> 0389; isolated from pickled onions</td>
<td>NODAI</td>
</tr>
<tr>
<td>5</td>
<td><em>L. plantarum</em> 1062</td>
<td>NODAI</td>
</tr>
<tr>
<td>6</td>
<td><em>L. plantarum</em> 1063</td>
<td>NODAI</td>
</tr>
<tr>
<td>7</td>
<td><em>L. plantarum</em> 1064</td>
<td>NODAI</td>
</tr>
<tr>
<td>8</td>
<td><em>L. plantarum</em> 1067T</td>
<td>NODAI</td>
</tr>
<tr>
<td>9</td>
<td><em>L. plantarum</em> 1078</td>
<td>NODAI</td>
</tr>
<tr>
<td>10</td>
<td><em>L. plantarum</em> 1757; isolated from Japanese pickled cabbage</td>
<td>NODAI</td>
</tr>
<tr>
<td>11</td>
<td><em>L. plantarum</em> 1832; isolated from Tai pickled cabbage</td>
<td>NODAI</td>
</tr>
<tr>
<td>12</td>
<td><em>L. plantarum</em> 1834; isolated from Tai pickled cabbage</td>
<td>NODAI</td>
</tr>
<tr>
<td>13</td>
<td><em>L. plantarum</em> FSCT-9; isolated from tomato</td>
<td>FATU</td>
</tr>
<tr>
<td>14</td>
<td><em>L. plantarum</em> FSCM-1; isolated from melon</td>
<td>FATU</td>
</tr>
<tr>
<td>15</td>
<td><em>L. plantarum</em> FSCM1-11; isolated from melon</td>
<td>FATU</td>
</tr>
<tr>
<td>16</td>
<td><em>L. plantarum</em> FSCM2-12; isolated from melon</td>
<td>FATU</td>
</tr>
</tbody>
</table>
SpeedVac® System ISS110; Savant Instruments Inv., NY, USA), and the residual fraction was dissolved in 2.0 ml of distilled water. The concentrated vitamin B2 extracts (1 ml), authentic vitamin B2 (50 ug/ml), and pseudo-vitamin B2 (50 ug/ml) were spotted onto a silica gel 60 thin-layer chromatography (TLC) sheet and developed in the dark using 2-propanol/NH4OH (28%)/water (7:1:2 v/v) at 25℃. After drying, the TLC sheet was overlaid with agar containing basal medium pre-cultured with E. coli 215 and incubated at 37℃ for 20 h. Next, the gel plate was sprayed with methanol solution containing 2,3,5-triphenyltetrazolium salt. Red coloration served as an indicator of E. coli growth and, thus, the presence of vitamin B2 compounds in the sample.

4. Identification of vitamin B2 compounds using liquid chromatography (LC)/electrospray ionization (ESI)-tandem mass spectrometry (MS/MS)

Vitamin B2 extracts (5 ml) were partially purified using a Sep-Pak® Plus C18 cartridge that had been washed with 5 ml of 75% (v/v) ethanol, equilibrated with 5 ml of distilled water, and washed with 5 ml of distilled water. Vitamin B2 compounds were eluted through the cartridge using 2 ml of 75% (v/v) ethanol. Eluates were evaporated to dryness under reduced pressure and subsequently dissolved in 10 ml of distilled water. Next, these fractions were loaded onto an immunoaffinity column (EASI-EXTRACT®; vitamin B2 Immunoaffinity Column [P 80] R-Biopharm AG, Darmstadt, Germany), and the compounds were purified according to the manufacturer’s protocol.

Purified vitamin B2 compounds were then dissolved in 0.1% (v/v) acetic acid and filtered through a Nanosep MF centrifuge device (0.4 μm; Pall Corp., Tokyo, Japan) to remove small particles. Subsequently, 2-μl aliquots of filtrates were analyzed using LC-MS ion trap/time-of-flight system coupled to Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Purified samples were then injected into an Inert-Sustain C18 column (3 μm, 2.0 × 100 mm; GL Science, Tokyo, Japan) equilibrated with 85% solvent A (0.1% (v/v) acetic acid) and 15% solvent B (100% methanol) at 40℃. Corrinoids were eluted using a linear gradient of methanol at a flow rate of 0.2 ml/min as follows: 15% solvent B for 5 min, solvent B levels increased from 15% to 90% over a period of 6 min, and solvent B levels decreased from 90% to 15% over a period of 5 min. The ESI-MS conditions were determined by injecting corrinoids into the MS detector, and the optimal parameters for detecting the parent and daughter ions of corrinoid compounds were identified. The ESI-MS system was operated in the positive ion mode, and argon was used as the collision gas. The identities of vitamin B2 (m/z 678.2914) and pseudo-vitamin B2 (m/z 672.7749) as [M + 2H]^+ were confirmed by comparing the observed molecular ions and retention times.

5. Lactobacillus growth experiments

A variety of Lactobacillus strains that were isolated either from freshly harvested plants or pickled vegetable products were pre-cultured by standing culture at 37℃ in MRS broth (Difco™ Lactobacilli MRS broth, Becton, Dickinson and Company, NJ, USA) and inoculated into a vitamin B2-deficient Lactobacillus medium or cabbage-extract medium. Vitamin B2 assay medium (Nissui Pharmaceutical Co., Ltd.) was used as the vitamin B2-deficient Lactobacillus medium.

Lactobacillus cells were grown in standing cultures at 37℃ in each of the test medium. Bacterial growth was spectrophotometrically measured by recording absorbance readings at 600 nm every 2h. Lactobacillus cells grown for 24 h were collected by centrifugation at 15,000 g for 10 min, washed twice with sterilized saline solution, and weighed. The Lactobacillus cell pellets were subjected to sonic oscillation to break the cells, and vitamin B2 content was evaluated as described above.

6. Preparation of sauerkraut via the addition of L. plantarum FSCM2-12

Commercially available fresh cabbage (approximately 250 g) was cut into thin strips and NaCl (5 g) was added and mixed well. L. plantarum FSCM2-12 (Table 1) was cultured in 100 ml of cabbage-extract medium at 37℃ for 24 h. The bacterial cells were collected by centrifugation at 15,000 g for 10 min before adding to the cabbage at concentrations of 0% (control), 0.1% (w/w; 0.25 g wet weight), 0.2% (w/w; 0.5 g wet weight), and 0.5% (w/w; 1.25 g wet weight). The L. plantarum FSCM2-12-treated cabbage was incubated for 3 days at 25℃ and used as sauerkraut. Each prepared sauerkraut sample was stained well and its vitamin B2 content was assayed as described above.

7. Statistical analyses

Biomass, vitamin B2 content in L. plantarum strains, and vitamin B2 content of sauerkraut
products with or without *L. plantarum* FSCM 2-12 were analyzed using one-way ANOVA with Tukey’s multiple comparison test. Analyses were performed using GraphPad Prism® for Windows, version 5.03 (GraphPad Software Inc., La Jolla, CA). All data are expressed as mean ± SEM, and *p* < 0.05 was considered statistically significant.

**Results and discussion**

1. Vitamin B₁₂ content of sauerkraut products

   In this study, the vitamin B₁₂ content of four types of sauerkraut (pickled cabbage) products were determined using the *L. delbrueckii* ATCC 7830 microbiological assay method (Table 2). Samples A, B, and C contained traces of vitamin B₁₂ (<0.1 µg/100 g wet weight), in line with values published in the Nutrient Databases. In contrast, sample D contained approximately 0.5 µg vitamin B₁₂/100 g wet weight, a significantly higher vitamin B₁₂ level.

2. LC/ESI-MS/MS analysis

   To determine whether the corrinoids present in sauerkraut sample D were vitamin B₁₂ or inactive corrinoids, such as pseudo-vitamin B₁₂, or mixtures, a sample D extract was purified using a vitamin B₁₂ immunoaffinity column and subsequently analyzed using LC/ESI-MS/MS. Authentic vitamin B₁₂ and pseudo-vitamin B₁₂ were eluted as peaks with retention times of 7.48 min and 7.35 min, respectively (Fig.1-A and D). The mass spectrum of authentic vitamin B₁₂ exhibited a prominent doubly charged ion with an *m/z* of 672.7756 ([M + 2H]⁺) (Fig.1-B). The calculated exact mass from the formula of vitamin B₁₂ (C₉₈H₆₈CoN₉O₈P) was 1354.5674, and the isotope distribution data illustrated that vitamin B₁₂ was the major doubly charged ion under these LC/ESI-MS/MS conditions. Pseudo-vitamin B₁₂, when analyzed, had a calculated exact mass of 1343.5375 ([C₉₇H₆₇CoN₉O₈P]⁺) and a prominent doubly charged ion with an *m/z* of 672.7756 ([M + 2H]⁺) (Fig.1-E). The MS/MS spectrum of authentic vitamin B₁₂ indicated that its dominant ion at *m/z* 359.0992 was attributable to the nucleotide moiety α-ribozale-5'-phosphate ([C₉₆H₆₄CoN₈O₇P]⁺, exact mass of 358.0929). Similarly, the MS/MS spectrum of pseudo-vitamin B₁₂ indicated that the dominant ion at *m/z* 348.0697 was attributable to a nucleotide moiety (Fig.1-F).

   Purified vitamin B₁₂ compounds from sauerkraut sample D were eluted as two ion peaks with *m/z* 672.7749 and 678.2914 at retention times of 7.35 and 7.48 min, respectively (Fig.2-A). The mass spectrum of the ion peak at *m/z* 672.7749 and retention time of 7.35 min illustrated that the doubly charged ion was formed at *m/z* 672.7725 (Fig.2-B). The MS/MS spectrum of this compound was identical to that of pseudo-vitamin B₁₂ (Fig.2-C). Moreover, the mass spectrum of the ion peak with a retention time of 7.48 min revealed that the doubly charged ion was formed at *m/z* 678.2901 (Fig.2-D). Finally, the MS/MS spectrum of the ion peak at *m/z* 678.2901 was identical to that of vitamin B₁₂ (Fig.2-E). These spectral data indicate that sauerkraut sample D mainly contained pseudo-vitamin B₁₂, an inactive corrinoid compound, and that none of these commercially available sauerkraut products are suitable sources of vitamin B₁₂.

3. Effects of cabbage extract on the growth and vitamin B₁₂ content in *Lactobacillus* strains isolated from vegetables and fruits and their products

   To create vitamin B₁₂-enriched sauerkraut product, 16 *Lactobacillus* strains isolated from vegetables and fruits, and their products were selected and cultured for 24 h in cabbage-extract medium. All strains displayed vitamin B₁₂-autotrophy because they grew well in vitamin B₁₂-deficient *Lactobacillus* medium (data now shown). However, *L. plantarum* 0389, 1078, 1757, 1832, 1834, FSCT-9, FSCM-1, FSCM 2-11, and FSCM 2-12 (Table 1) were grown in cabbage-extract medium, unlike the remaining *Lactobacillus* strains (Fig.3). Fig.4 shows the biomass and vitamin B₁₂ content in these *Lactobacillus* cells grown for 24 h in cabbage-extract medium. *L.
Plantarum FSCM2-12 produced the highest biomass (approximately 0.81 ± 0.09 g wet weight) and had the greatest vitamin B₁₂ content (approximately 46.8 ± 11.4 ng/g wet weight) among the tested cells. This vitamin B₁₂ content was much higher (13 ng of vitamin B₁₂/g dry biomass) than that in L. plantarum.
isolated from an Indian traditional fermented food. The corrinoid compounds formed by *L. plantarum* FSCM2-12 were separated using silica gel 60 TLC and visualized using *E. coli* 215 bioautography (Fig. 5). An extract of *L. plantarum* FSCM 2-12 cells revealed a positive spot with an *R* value of 0.6, which was identical to that of authentic vitamin B12 but not that of pseudo-vitamin B12. Recently, the probiotic properties of *L. plantarum* FSCM2-12 were further characterized.

In the present study, using a microbiological assay, *L. corynformis* 1051T and 1638T grown for 24h in MRS broth were found to contain 103.3 ± 16.4 and 75.5 ± 8.1 ng of vitamin B12/g wet weight, respectively. However, the corrinoid compounds found in these bacteria were identified as pseudo-vitamin B12 by *E. coli* 215 bioautography (data not shown), in concert with previous results reported.
Sixteen *Lactobacillus* strains isolated from plants and their products were pre-cultured in MRS medium (Difco™), inoculated in cabbage-extract medium, and incubated for 24 h. Bacterial growth was estimated by measuring the absorbance at 600 nm at 2 h time points. The *L. plantarum* strains corresponding to sample numbers are listed in Table 1. A O (No. 1), (No. 2, 3, and 4); B O (No. 5), (No. 6, 7, and 8); C O (No. 9), (No. 10, 11, and 12); D O (No. 13), (No. 14, 15, and 16). Data represent the mean ± SEM of three independent experiments.

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4. Vitamin B12 content of sauerkraut products fermented with or without *L. plantarum* FSCM2-12 cells

To generate vitamin B12-enriched sauerkraut products, fresh cabbage (approximately 250 g) was fermented for 3 days at 25°C in vessels with various concentrations (0%, no vitamin B12 content; 0.1% [w/w], 15 ng of vitamin B12; 0.2% [w/w], 31 ng of vitamin B12; and 0.5% [w/w], 78 ng of vitamin B12) of *L. plantarum* FSCM2-12 cells as a starter (Fig. 6). The sauerkraut products prepared without *L. plantarum* FSCM2-12 (spontaneous fermentation by concomitant lactic acid bacteria; control) contained vitamin B12 at approximately 0.1 μg/100 g wet weight, which closely matches the vitamin B12 levels of the commercially available sauerkraut products A, B, and C. Treatment of the fresh, 3-day sauerkraut with *L. plantarum* FSCM 2-12 was significantly increase the vitamin B12 content in a concentration-dependent manner. The 0.5% (w/w) *L. plantarum* FSCM 2-12-supplemented sauerkraut products contained vitamin B12 at approximately 0.3 μg/100 g wet weight, which is three times greater than that of the control. Our results suggest that *L. plantarum* FSCM 2-12 can be used as a starter for the preparation of vitamin B12-enriched sauerkraut products.
In order to prepare commercially available vitamin B₁₂-enriched sauerkraut products, fermentation conditions must be detailed and optimized.

Author Contributions

T.S. and K.F. assayed the vitamin B₁₂ content of sauerkraut samples and analyzed corrinoid compounds using \textit{E. coli} 215 bioautography. S.T. analyzed corrinoid compounds using LC/ESI-MS/MS and interpreted the results. T.B., Y.K., and Y.T. performed growth experiments for lactic bacteria. T.A., J.S., and T.F. contributed to the design of the project and discussed the results. T.B., Y.Y., and F.W. designed the experiments, interpreted the results, and wrote the manuscript. All authors commented on the manuscript and approved the final version.

Notes

The authors declare no competing financial interests.

References


