Production Potency of Folate, Vitamin B₁₂, and Thiamine by Lactic Acid Bacteria Isolated from Japanese Pickles

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We investigated the extracellular production of folate, vitamin B₁₂, and thiamine in cultures of lactic acid bacteria (LAB) isolated from nukazuke, a traditional Japanese pickle, and the relationships between the vitamin production and such properties of LAB as tolerance to salts, ethanol, etc. Among the 180 isolates of LAB, two strains of Lactobacillus (Lb.) sakei and a strain of Lb. plantarum extracellularly produced high levels of folate (about 100 μg/L). A strain of Lb. coryniformis and one of Lb. plantarum produced about 2 μg/L of vitamin B₁₂, although the level was not high. No isolates produced a high level of thiamine. The type cultures of LBA (53 strains) did not show any higher production of these vitamins. Some isolates showed tolerance to high concentrations of salts and alcohol, and low initial pH. No significant relationships between folate or vitamin B₁₂ productions and these properties of LAB were apparent.

Key words: lactic acid bacteria; nukazuke; folate; vitamin B₁₂; thiamine

Lactic acid bacteria (LAB) are useful microbes for producing food and have been used for a variety of such traditional fermented foods as cheese, miso, and pickles. LAB have also been known as probiotics which improve intestinal microflora and have shown beneficial effects on human health.¹² Nukazuke is a type of Japanese pickle, made of vegetables and salt in a fermented rice bran bed (nukadoko). Nukadoko can be aged for a number of years without any spoilage, despite its repetitive natural fermentation without sterilized rice bran and vegetables. This aging process tends to enhance its quality, e.g., a favorable flavor and well-balanced microbiota.³⁴ Imai et al. have reported that the microbiota of nukadoko mainly consisted of such LAB species as Lactobacillus (Lb.) plantarum, Pediococcus pentosaceus, and Tetragnococcus halophilus.³⁵ Nakayama et al. have reported that such Lb. species as Lb. acidifanae-like bacteria and Lb. acetotolerans were found to be predominant in long-aged nukadoko as unculturable and slowly growing lactobacilli.³⁵

Deficiencies of folate and vitamin B₁₂ may be a public health problem affecting millions of people according to a recent World Health Organization review;⁶ for example, a low folate and vitamin B₁₂ status increases the risks of anemia and birth defects, and these risks are reduced by supplying these vitamins.⁷³ Microbial vitamin production is a convenient strategy to achieve natural enrichment of vitamins in fermented foods, notably from vegetable sources. Such an LAB as Lb. plantarum is known for folate production in a high concentration,⁹¹⁰ while Lb. reuteri is known for vitamin B₁₂ production in a high concentration.¹¹ Although some authors have reported folate-productive LAB strains isolated from radish, frozen peas, yogurt, cheese¹² and cow’s milk,¹³ and vitamin B₁₂-productive strains isolated from sourdough¹¹ and goat’s milk,¹⁴ there are no data available concerning LAB isolated from traditional Japanese pickles. Furthermore, although it has been reported that bifidobacteria produced high levels of thiamine,¹⁵ few studies have investigated the thiamine production of LAB. There has also been no report on the relationship between the production of vitamins and such properties of LAB as tolerance to salts and pH value.

The purpose of this study is first to investigate the potency for the extracellular production of folate, vitamin B₁₂, and thiamine by LAB isolated from nukazuke, and second to examine the relationships between the vitamin production and such properties of LAB as the tolerance to NaCl, NaNO₂, ethanol, temperature, and pH value.

Materials and Methods

Isolation of LBA. A total of 146 nukazuke samples, traditional Japanese pickles, were used for isolating LAB. The nukazuke samples, including cucumber, radish, eggplant, turnip, carrot, cabbage, and Chinese cabbage, were purchased from 101 retail markets in the Kanto-area of Japan from 2004 to 2006. Each sample (about 10 g) was homogenized in 10 times its weight of sterile 0.85% NaCl by a Masticator PS device (GSI Creos, Tokyo, Japan). After appropriate dilutions were made with 0.85% NaCl, 0.1 mL of a diluted sample was mixed with 15 mL of de Man-Rogosa-Sharpe (MRS) agar (Merck

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Abbreviations: LAB, lactic acid bacteria; Lb., Lactobacillus; PBS, phosphate-buffered saline; MRS, de Man-Rogosa-Sharpe; OD660, optical density at 660 nm
The thiamine concentration of each culture supernatant was determined after 20 h of incubation. The thiamine determination was therefore performed as described by Torriani et al. 

DNA isolation. The strains showing higher production levels of folate or vitamin B12 were identified. Bacterial DNA was extracted with InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) by following the manufacturer’s instructions.

Sequence analysis. One microgram of the purified pGEM plasmid was used for a sequence analysis of the cloned 16S rDNA fragments. Nucleotide sequencing of cloned DNA was performed by the dideoxy chain termination method with primer walking, using the BigDye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems) and the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sequence similarity searches were performed in the GenBank data library by using the FASTA program.

Fermentation of carbohydrate. Although Lb. sakei and Lb. curvatus were closely related in the 16S rRNA sequence-coding region, these species differed in the fermentation of melibiose. Melibiose fermentation was therefore examined by using the classical microtube fermentation test described by Klein et al. 

Multiplex PCR. The Lb. plantarum, Lb. pentosus, and Lb. paraplantarum species are genotypically closely related in the 16S rRNA sequence-coding region. A recA-Nested multiplex-PCR assay was therefore performed as described by Torriani et al. 

Species-specific PCR assay. Specific PCR primers for Lb. plantarum and Lc. cryoniforms were designed on the basis of the 16S rDNA genes of Lb. plantarum (accession no. EF536363, position 1-1530) and
Results

Isolation of LAB from nukazuke

A total of 180 LAB strains were isolated from 146 nukazuke samples. The number of LAB was detected in the range of $10^7$–$10^9$ CFU/g, the number of LAB in some samples exceeding $10^9$ CFU/g. 

Extracellular folate production

A total of 233 LAB strains (180 strains isolated from Nukazuke and 53 LAB reference strains) were screened for their ability to grow in the folate-free medium that did not contain any folate. As a result, 139 strains did not grow in the absence of folate, while 96 strains (73 isolates and 23 reference strains) grew after 24 h of incubation. As the latter strains were considered to be folate auxotrophs, the extracellular folate was analyzed. Less than 20 μg/L was treated as a trace for folate production, while concentrations over 20 μg/L were considered positive. The strains which produced folate were further tested for their ability to grow in the folate-free medium that contained 10 μg/L folate. Eight strains produced folate, and some of their properties are shown in Table 1.

Table 1. Higher Folate- and Vitamin B$_{12}$-Producing Lactic Acid Bacteria Isolated from Nukazuke and Some Properties

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Vitamin production</th>
<th>Growth under the conditions of</th>
<th>Folate (+Con)</th>
<th>OD$_{660}$</th>
<th>Vitamin B$_{12}$ (+Con)</th>
<th>OD$_{660}$</th>
<th>NaCl (%)</th>
<th>NaNO$_2$ (%)</th>
<th>Ethanol (%)</th>
<th>Temperature (°C)</th>
<th>Initial pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lb. sakei</em> CN-3</td>
<td>101 ± 10</td>
<td>&lt;0.1</td>
<td>6</td>
<td>1.0</td>
<td>5.0</td>
<td>15–37</td>
<td>5–8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. sakei</em> CN-28</td>
<td>107 ± 6</td>
<td>0.27 ± 0.03</td>
<td>&lt;6</td>
<td>1.0</td>
<td>5.0</td>
<td>25–37</td>
<td>5–7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> CN-49</td>
<td>108 ± 9</td>
<td>0.20 ± 0.03</td>
<td>8</td>
<td>2.0</td>
<td>10.0</td>
<td>15–37</td>
<td>3–8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> CN-225</td>
<td>trace</td>
<td>1.8 ± 0.1</td>
<td>7</td>
<td>&gt;3.0</td>
<td>2.5</td>
<td>15–37</td>
<td>6–7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. corynformis</em> CN-229</td>
<td>81 ± 12</td>
<td>2.0 ± 0.1</td>
<td>6</td>
<td>&gt;3.0</td>
<td>5.0</td>
<td>15–37</td>
<td>6–7</td>
<td></td>
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</tr>
</tbody>
</table>

Folate concentrations of cultured supernatants of a folate-free medium after 24 h of incubation were determined ($n = 3$). trace, <2.4 μg/mL of folate. Vitamin B$_{12}$ concentrations of cultured supernatants of a vitamin B$_{12}$-free medium after 48 h of incubation were determined. Growth of each strain (OD$_{660} > 0.1$) was observed after 24 h of incubation under each growth condition.

Fig. 1. Typical Time-Course Characteristics for the Extracellular Folate Production by *Lactobacillus plantarum* CN-49 (A) and for the Extracellular Vitamin B$_{12}$ Production by *Lactobacillus casei* CN-225 (B).

CN-49 was incubated at 37 °C for 24 h in a broth containing folic acid assay medium, and CN-229 was incubated at 37 °C for 48 h in a broth containing the Nissl B$_{12}$ basal medium. Folate was analyzed with (+Con) and without (−Con) a conjugase treatment.

*Lb. corynformis* (accession no. HM217943, position 436–1018). The primers specific for *L. plantarum*, LplaF (5'-TCAGGTATTGACGGTATTTAACCA-3'; forward) and LplAR (5'-TTCCGTTAGC-TTCGATTAAAA-3'; reverse), and for *Lb. corynformis*, LcoryF (5'-TGGAGAAAGACGGGACTAGA-3'; forward) and LcoryR (5'-GCTGTATCTCTACAGGGTCAAGG-3'; reverse) were respectively used. The designed primers respectively yielded 505 bp and 583 bp amplified products with *L. plantarum* and *Lb. corynformis*. The samples were amplified by the following program: pre-denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min.

Vitamin B$_{12}$-Producing Lactic Acid Bacteria Isolated from Nukazuke and Some Properties

Table 1. Higher Folate- and Vitamin B$_{12}$-Producing Lactic Acid Bacteria Isolated from Nukazuke and Some Properties

A total of 180 LAB strains were isolated from 146 nukazuke samples. The number of LAB was detected in the range of $10^7$–$10^9$ CFU/g, the number of LAB in some samples exceeding $10^9$ CFU/g. *Lb. sakei* CN-3 utilized melibiose, arabinose, and sucrose as carbon sources, while *Lb. plantarum* CN-23 utilized lactose, fructose, and galactose as carbon sources. The highest folate production of about 100 μg/L in a folate-free medium. Among the reference strains, one strain of *Lb. sakei* and *Lb. plantarum* showed relatively high folate production (50–60 μg/L), although this level did not reach that of the three isolates from nukazuke.

The sequences of 16S rRNA were determined to identify these three strains. For CN-3, the sequence data of about 500 bp for the 16S rRNA gene showed 98.9, 98.7, and 97.1% respective homology to *Lb. sakei*, *Lb. curvatus*, and *Lactococcus lactis*. CN-3 was a rod, so *Lactococcus lactis* was excluded. The utilization of melibiose, arabinose and sucrose was examined to discriminate between the two remaining species. CN-3 utilized melibiose, arabinose, and sucrose as carbon sources. *Lb. sakei* did, while *Lb. curvatus* did not, CN-3 therefore being identified as *Lb. sakei*. CN-28 was similarly identified as *Lb. sakei*. For CN-49, the sequence data of about 500 bp for the 16S rRNA gene showed 99.8, 99.6, and 99.5% respective homology to *Lb. plantarum*, *Lb. paraplantarum* and *L. paraplenarum*. A recA nested multiplex PCR analysis was conducted to discriminate among these three species. The results showed the band of CN-3 to be identical with that of *Lb. plantarum* and different from those of *Lb. pentosus* and *Lb. paraplantarum*. CN-49 was thus identified as *Lb. plantarum*. Some properties of these strains are shown in Table 1. *Lb. sakei* CN-3, *Lb. sakei* CN-28, and *Lb. plantarum* CN-49 respectively produced 101, 107, and 108 μg/L of folate in a folate-free medium after 24 h of incubation. They grew in the presence of 6% of NaCl, 1% of NaNO$_2$, and 5% ethanol. Figure 1A shows typical time-course characteristics for folate production by *Lb. plantarum* CN-49. Folate was produced a little behind the growth, its concentration reaching the maximum in the stationary phase. The folate formed by CN-49 was the conjugate type of pteroylpolyglutamate, the amount...
of folate being considerably less without the conjugase treatment (Fig. 1A).

**Extracellular vitamin B$_{12}$ production**

A total of 233 LAB strains (180 strains isolated from nukazuke and 53 LAB reference strains) were screened for their ability to grow in a vitamin B$_{12}$-free medium. As a result, 77 strains did not grow in the absence of vitamin B$_{12}$, while 156 strains (123 isolates and 33 reference strains) grew after 24 h of incubation. Since the latter strains were considered to synthesize vitamin B$_{12}$ in their cells, the extracellular vitamin B$_{12}$ was analyzed. Less than 0.20 μg/L was treated as a trace. The vitamin B$_{12}$ concentration of the cultured broth of 83 strains that showed concentrations of more than a trace ranged from 0.2 to 2.0 μg/L. Two strains (CN-229 and CN-225) showed the highest production of vitamin B$_{12}$ among them. CN-229 and CN-225 produced about 2 μg/L of vitamin B$_{12}$ in the vitamin B$_{12}$-free medium. Among the reference strains, one strain each of Pediococcus pentosaceus and Lb. brevis showed a relatively high production (0.4–0.6 μg/L), although this level was lower than that of the isolates from nukazuke.

These two strains were next identified. CN-229 was a rod and identified as *Lb. coryniformis* by the sequence data of about 500 bp for the 16S rRNA gene. Furthermore, a single amplification product of 583 bp was obtained with the LcoryF and LcoryR primers specific for *Lb. coryniformis*, while no amplification product was detected with the Lplaf and Lplar primers specific for *Lb. coryniformis*, while no amplification product was detected with the Lplaf and Lplar primers specific for *Lb. coryniformis*, while no amplification product was detected with the LcoryF and LcoryR primers specific for *Lb. coryniformis*.

Some properties of these strains are shown in Table 1. *Lb. coryniformis* CN-229 and *Lb. plantarum* CN-225 respectively produced 1.8 and 2.0 μg/L of vitamin B$_{12}$ in a vitamin B$_{12}$-free medium after 48 h of incubation. The alkaline heat-resistant factor was negligible. They grew in the presence of 6% NaCl, >3% NaNO$_{2}$, and 2.5% ethanol. They did not grow pH < 6. *Lb. coryniformis* CN-229 produced 81 μg/L of folate in a folate-free medium, while *Lb. plantarum* CN-225 produced little. Figure 1B shows typical time-course characteristics for the vitamin B$_{12}$ production by *Lb. coryniformis* CN-229. Vitamin B$_{12}$ was produced behind the growth, its concentration reaching the maximum after 48 h of incubation. Lb. plantarum CN-225 showed similar patterns of growth and vitamin B$_{12}$ production to those of *Lb. coryniformis* CN-229.

**Extracellular thiamine production**

A total of 230 LAB strains (183 strains isolated from nukazuke and 53 LAB reference strains) were screened for their ability to grow in the thiamine-free medium. The results show that 76 strains (50 isolates and 26 reference strains) grew in the absence of thiamine after 24 h of incubation. The extracellular thiamine was analyzed for all these strains: all LAB strains produced only a level at the detection limit or no thiamine (0–0.18 μg/L) and we could not find any high-thiamin-producing LBA. We then examined the intracellular thiamine for 11 strains (6 reference strains and 5 isolates) showing good growth in the thiamine-free medium. Table 2 shows the results for the reference strains. These cells contained thiamine at the level of only 0.1–0.2 ng/mg fresh-weight of cells, although this level was several times higher than that of the extracellular thiamine. The results for the five isolate strains were similar to those for the reference strains. It seems that LBA produced about 10 μg/L of thiamine, and 80–90% of this amount being in the cells and 10–20% being released into the medium.

**Effects of NaCl, NaNO$_{2}$, ethanol, temperature, and initial pH on growth of the LAB isolates**

The effects of NaCl, NaNO$_{2}$, ethanol, temperature, and initial pH on the growth of the LAB strains isolated from nukazuke were examined for 74 randomly selected strains among the 180 isolates. Most strains showed tolerance to 6–7% of NaCl, 1–2% NaNO$_{2}$, and 5% ethanol (Fig. 2A–C). All the LAB strains grew at 25–37°C, but there was little LBA growth when incubated at 45°C (Fig. 2D). Although most strains grew at an initial pH value of 5–7, 15 strains of LAB grew at low pH (3 or less) (Fig. 2E). Table 3 shows the properties of several peculiar strains. Excepting the strain showing growth in the presence of 3% NaNO$_{2}$, they grew in a relatively high concentration of NaCl (8–10%). Three strains grew in the presence of 10% ethanol, one strain grew at a temperature higher than 45°C, and two strains grew at a low initial pH value of 3.

**Relationship between the production of folate or vitamin B$_{12}$ and the species of LAB**

To examine any relationship between the species of LBA and the production of folate and vitamin B$_{12}$, the
production of 63 strains (53 reference strains and 10 strains isolated from nukazuke) was plotted for each species (Fig. 3). The highest extracellular folate concentrations were observed for \textit{Lb. coryniformis}, \textit{Lb. plantarum}, and \textit{Lb. sakei}. The \textit{Lb. plantarum} species showed a wide range of folate production, with negative production for 6 strains and positive production for 6 strains (35–101 mg/L of folate). The highest extracellular vitamin B$_{12}$ yield was only observed only for a strain of \textit{Lb. coryniformis} and \textit{Lb. plantarum} isolated from nukazuke.

**Relationship between the production of folate or vitamin B$_{12}$ and some properties of LAB**

We checked if there was any relationship between the production of vitamins and such properties of LAB as the tolerance to NaCl, NaNO$_2$, ethanol, temperature, and initial pH value. Table 1 and Fig. 2 shows that the strains with the higher production levels of folate and vitamin B$_{12}$ did not have any specific properties in terms of tolerance to NaCl, NaNO$_2$, ethanol, temperature, and initial pH value. The strains having relatively strong tolerance to salts, ethanol, and acidity shown in Table 2 did not show any higher production of vitamins. There was thus no significant relationship between the production of folate and vitamin B$_{12}$ and these properties of LAB.

**Discussion**

We isolated LAB from nukazuke in this study and investigated the extracellular production of folate,
vitamin B$_{12}$, and thiamine. The number of LAB detected in nukazuke was in the range of $10^7$–$10^9$ CFU/g in this study. Lee et al. have reported that the number of LAB isolated from kimchi was detected in the range of $10^7$–$10^9$ CFU/g. Tamang et al. have reported that the number of LAB isolated from traditionally fermented vegetable products of the Eastern Himalayas was detected in the range of $10^7$–$10^9$ CFU/g. Our result was similar to those figures.

Three-strains with the highest yield of extracellular folate were identified in this study. One strain of _Lb. plantarum_ and two strains of _Lb. sakei_ produced about 100 µg/L of folate in a folate-free medium (Table 1). Hugenschmidt et al. have recently reported that 96% of _Lb. plantarum_ strains produced extracellular folate. However, only 50% of _Lb. plantarum_ strains produced extracellular folate in this present study. Folate was produced at different levels by the different LAB strains, folate production seeming to depend on the strains but not on the species. It has recently been shown that _Lb._ species could produce folate at different levels; 41 µg/L and 131 µg/L by _Lb. brevis_, 19.8 µg/L by _Lb. acidophilus_, 125 µg/L and 21.1 µg/L by _Lb. reuteri_, 46 µg/L by _Lb. lactis_, 66.6 µg/L by _Lb. crustorum_, and 397 µg/L by _Lb. plantarum_. To our knowledge, the highest folate production by a natural strain has been reported to be ca. 400 µg/L by a _Lb. plantarum_ isolate from salami. The maximum folate concentration in our study was about 100 µg/L, significantly less than the 400 µg/L maximum. It will be necessary to investigate and optimize the fermentation conditions and identify which kinds of folate derivative are formed.

The extracellular vitamin B$_{12}$ production was also examined in this study. Two strains showed the highest extracellular production level of vitamin B$_{12}$ among those examined, although this level was relatively low: it has been reported that _L. reuteri_ CRL1098 intracellularly produced 500 µg/L of vitamin B$_{12}$, although there was no comment on the extracellular production level by this strain. _L. reuteri_ has been reported to metabolize glycerol in a vitamin B$_{12}$-free medium, this being the first indirect evidence for the presence of intracellular cobalamin. The sequenced genome data has shown that _L. reuteri_ contained the genes for cobalamin biosynthesis and propanediol utilization (cob-pdu). L. reuteri JCM 1112 has a unique cluster of 58 genes for the biosynthesis of reuterin and cobalamin. This 58-gene cluster is apparently inserted into the conserved region, suggesting that the cluster represents a genomic island acquired from an anomalously source. _L. cornimformis_ has also been reported to produce a cobalamin-type of compound. The strains of _Lb. cornimformis_ and _Lb. plantarum_ were identified in this study as the highest extracellular producers of vitamin B$_{12}$. The cob-pdu cluster may have spread to lacticobacilli other than _Lb. cornimformis_. Further studies on the distribution or spread of vitamin B$_{12}$-producing LAB might be needed. We could not find any LAB highly producing thiamine. It seems that LBA could neither extracellularly nor intracellularly produce substantial thiamine.
To our knowledge, this is the first study to evaluate the potency for the extracellular production of folate, vitamin B_{12}, and thiamin by LAB isolated from the traditional Japanese pickle, nukazuke. Although some of the LBA isolates produced a higher level of extracellular folate and lower level of vitamin B_{12}, no highly-productive strains of vitamin B_{12} and thiamin were found. There was no significant relationship between the extracellular production of folate or vitamin B_{12} and such properties of LAB as tolerance to salts, ethanol, temperature, and initial pH. It will be necessary to optimize the conditions for vitamin production and to develop a method utilizing folate–producing LBA in foods or beverages.

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