Quality Improvement of Miso and Soy Sauce by Lactic Acid Bacteria: Inhibition of Spoilage Bacteria and Browning†

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It is well-known that some lactic acid bacteria produce bacteriocins, which have been defined as proteinaceous substances with bactericidal activity against a limited range of bacteria. Nisin, produced by some lactococci, has bactericidal effects against gram-positive bacteria such as Bacillus and Clostridium sp. Nisin is not sporicidal but prevents the growth of germinated bacterial spores. Nisin is the only bacteriocin that has been commercially exploited as a food-additive, because of its degradation in the human alimentary canal by digestive proteases. Applications of nisin or nisin-producing starter cultures have been developed for dairy foods, meat and fish products, and fermented beverages. On the other hand, the use of nisin or nisin-producing starter cultures to control spoilage bacteria in fermented seasonings like miso has not been described.

Miso, fermented soybean paste, is a very important seasoning in Japan or Asian countries. One of the most serious problems in miso fermentation is the proliferation of spoilage bacteria, especially spore-forming bacteria. Among these Bacillus subtilis proliferates rapidly during koji fermentation which is the process of culturing Aspergillus oryzae on cooked soybean (soybean miso), cooked rice (rice miso), or cooked barley (barley miso). B. subtilis not only antagonistically prevents the growth of Asp. oryzae but also produces off-flavors, resulting in serious quality problems in the product. Furthermore, since most of B. subtilis remain as spores, miso sometimes causes spoilage of processed foods seasoned with miso. In spite of many efforts, practically available methods to effectively prevent B. subtilis have not yet been developed. Bacteriocinogenic lactic acid bacteria, with their bacteriocins such as nisin would make them ideal candidates to prevent the growth of spoilage bacteria in fermented seasonings.

In this study, we examined the growth inhibition of B. subtilis by lactic acid bacteria, especially nisin-producing lactococci, and its application to miso fermentation as a starter culture to prevent growth of B. subtilis and improve miso quality. Furthermore, we also investigated the prevention of browning of miso by the use of lactic acid bacteria, because excessive browning is a serious problem causing miso to lose its commercial value.

1. Growth of lactic acid bacteria and growth
inhibition of B. subtilis in cooked soybeans

Most lactic acid bacteria (LAB) tested, such as Lc. lactis subsp. lactis IFO12007, Pediococcus cerevisiae DSM1955, Enterococcus casseliflavus ATCC14432, and Lactobacillus plantarum ATCC 14917, rapidly proliferated and reached greater than 10⁶ cells/g in cooked soybeans following 24 h of incubation. In general, LAB are complex auxotrophs and require nutrients such as sugars, vitamins, amino acids, etc. The substantial growth of LAB indicated that cooked soybeans contained enough nutrients and needed no supplements for the rapid growth of LAB.

To study growth inhibition of B. subtilis by nisin-producing LAB, cooked soybeans were fermented with Lc. lactis IF012007 (nisin-producing, salt-intolerant) at 30°C for 24 h and then inoculated with B. subtilis ATCC19659 at levels of 10⁶ to 10⁸ cells/g, followed by incubation at 30°C for 48 h. B. subtilis inoculated into the 24-h-lactic acid fermented soybeans was not detected as measured by the viable counts even immediately after inoculation. On the other hand, in the absence of LAB, B. subtilis rapidly proliferated and reached above 10⁹ cells/g in cooked soybeans after 24 h of incubation. The soybeans were markedly spoiled, producing ill-smelling compounds and slime. Based on these results, it could be concluded that soybeans fermented with nisin-producing LAB was so inhibitory that B. subtilis inoculated after lactic acid fermentation could no longer survive.

2. Growth inhibition of B. subtilis in soybean miso by nisin-producing lactococci

Soybean miso was prepared with nisin-producing lactococci by the method shown in the Fig.1. As shown in the Table 1, Lc. lactis IFO12007 rapidly proliferated and produced a nisin activity of 6.4 × 10⁴IU/g in the soybeans after 24 h of incubation. During koji fermentation, no B. subtilis was detected in the koji despite inoculation of 10⁶ cells/g, suggesting that the B. subtilis inoculated were immediately killed by the nisin accumulated in the lactic acid fermented soybeans. No B. subtilis were detected in miso at any stage during the 31 days of incubation. On the other hand, a large number of LAB were still present immediately after shikomi treatment. Then, LAB gradually decreased and subsequently disappeared in miso on day 24 of incubation. It was thought that Lc. lactis IFO12007 could not be kept alive under the high concentration of sodium chloride (11%), because of its salt-intolerant nature. The pH of miso after 31 days was 5.7 which was a typical value for common soybean miso. Nisin activity gradually decreased during aging and finally disappeared after 31 days. This disappearance of nisin in the miso is important for legal regulation in Japan, because nisin is not permitted for use as a food preservative. When soybean miso was prepared without LAB and with B. subtilis, B. subtilis proliferated at 2.4 × 10⁹ cells/g after 24 h and their spores of 1.4 × 10⁸ cells/g remained in the miso after 31 days of incubation (Table1). It was also confirmed that lactic
Table 1 Complete growth inhibition of *B. subtilis* by nisin-producing lactococci during fermentation of soybean miso and hyposalt miso

<table>
<thead>
<tr>
<th></th>
<th>Soybean miso (11% NaCl)</th>
<th>Nonsalt miso</th>
<th>Hyposalt miso</th>
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</thead>
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<tr>
<td></td>
<td>LA ferm</td>
<td>Koji ferm</td>
<td>Aging</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>–</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LAB</td>
<td>3.7×10⁶</td>
<td>2.8×10⁶</td>
<td>1.5×10⁶</td>
</tr>
<tr>
<td>Nisin (IU/g)</td>
<td>0</td>
<td>6.4×10⁶</td>
<td>8.0×10⁶</td>
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<tr>
<td>pH</td>
<td>6.2</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Control (no LAB)</td>
<td>–</td>
<td>1.3×10⁶</td>
<td>2.4×10⁶</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>–</td>
<td>6.3</td>
<td>6.4</td>
</tr>
<tr>
<td>pH</td>
<td>–</td>
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After the lactic acid fermentation with *Lc. lactis* IFO12007 (nisin-producer) for 24 h, the soybeans were inoculated with *Asp. oryzae* KBN967 together with 10⁶ cells/g of *B. subtilis* ATCC19659, before incubating at 30°C for 48 h as the koji fermentation step. Soybean miso containing zero %, 8%, and 11% sodium chloride was incubated at 30°C for 28 days for aging. ND: not detected. Nisin detection limit: 7.5 IU/g.

acid fermentation of soybeans with *Lc. lactis* IFO12007 did not affect koji fermentation and degradation of soybean proteins during aging of miso.

3. Hyposalting of soybean miso with nisin-producing lactococci

Nonsalt-miso and hyposalt-miso containing 8% sodium chloride were prepared with nisin-producing lactococci. *B. subtilis* inoculated at a level of 10⁶ cells/g was not detected in either nonsalt- or hyposalt-miso at any stage of incubation (Table 1). A large number of LAB were still present in nonsalt-miso after 17 days of aging, the pH of which decreased to 5.1. This value was slightly lower than that of normal salt-miso (11% sodium chloride). On the other hand, changes in the number of LAB and the pH of hyposalt-miso were almost the same as those for normal salt-miso. These results suggested that hyposalt-miso could be prepared without difficulty by the use of antibacterial starter cultures.

4. Prevention of browning of soybean miso with lactic acid bacteria

Color is one of the most important factors for miso. Since excessive brown-colored miso loses its commercial value, prevention or control of browning reactions is highly desirable. When soybean miso was prepared with *Lc. lactis* IFO12007 as a starter culture, the color of miso after 28 days of aging was perceptibly lighter than that of the control without LAB; Y% values (indicating brightness or whiteness) of the miso with and without LAB were of 23.1 and 18.5, respectively (Niwa et al., unpublished results). However, the lightening effect decreased with aging and was finally lost after 42 days (Y% values of the both miso were...
about 15.0). *Lc. lactis* IFO12007 dies after 21 days due to its salt-intolerant nature. The use of the mixed starter culture containing *Ec. casseliflavus* IFO12256 and *Tetragenococcus halophilus* which are both salt-tolerant and therefore remain alive during aging, was very effective on lightening the color of soybean miso; the Y% value was 26.0 after 42 days of aging. Furthermore, 3-deoxy-glucoosone amounts and fluorescent strength (340 nm and 430 nm) of the miso with the mixed starter culture were low, indicating prevention of browning reaction. On the basis of these results, it is concluded that the existence of viable LAB was effective in preventing the browning reaction in soybean miso.

5. Lactic acid starter cultures for fermented seasonings

Several characteristics are required of LAB as an antibacterial starter culture for soybean miso; 1) rapid growth and production of antibacterial substances in cooked soybeans or koji, 2) no inhibition against *Asp. oryzae*, 3) limited pH decrease, 4) salt intolerance, 5) no production of compounds which degrade the quality (smell, color, etc.), 6) non-pathogenic. It was known that some bacteria antagonistically prevent koji fermentation, resulting in reduced quality of the products. We have shown that some lactic acid bacteria, such as *L. plantarum* strongly suppress koji fermentation. *Lc. lactis* IFO12007, nisin-producing and salt-intolerant, satisfied all these characteristics necessary for soybean miso fermentation. Since nisin is inhibitory to LAB (bacteriocins produced by LAB are the most inhibitory against LAB), nisin-producing lactococci is expected to be effective on the inhibition of not only *B. subtilis* but also of undesirable LAB in soybean miso fermentation.

Hyposalting of foods is considered to be important to prevent human age-related or lifestyle-related diseases like hypertension. Hyposalting of fermented seasoning would be especially important, because high concentrations of sodium chloride are generally added to miso (11-13%) and soy sauce (18%) to prevent the growth of bacteria which cause spoilage. If prevention of bacteria can be achieved by lactic acid starter cultures, high concentrations of salt would not be necessary for miso fermentation. Furthermore, since protease activity can be maintained under low salt concentrations, hyposalting might accelerate proteolysis of soybean proteins, resulting in shortening of aging period during miso production. The method developed in this study is expected to be applicable to rice miso, soy sauce and tamari sauce fermentation, because fermentation procedures of these seasonings and their microbiological problems are substantially the same as those of soybean miso.

References