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Isolation and identification of psychrotrophic lactic acid bacteria in *godo*, the traditional fermented soy food in Japan

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***Godo* is a traditional fermented soy food made in Aomori prefecture, Japan. It is mainly made of soybeans, rice *koji*, and salt. Since *godo* ripens during the long and severe winter in northeast Japan, it is assumed that lactic acid bacteria inhabiting *godo* have cold tolerance. We aimed to investigate the presence or absence of psychrotrophic lactic acid bacteria in *godo*. The viable counts of estimated lactic acid bacteria ranged from 10^6 to 10^8 cfu/g. In addition, aerobic and anaerobic microorganisms were detected in four *godo* products though the microbial population differed from sample to sample. Twenty-two bacterial strains were able to be isolated from *godo*, and all of the isolated strains were Gram-positive and catalase-negative. Some of the isolates grew well at 10°C. The carbohydrate fermentation profile of the selected three strains was determined by API50 CHL analysis. These strains were identified as *Leuconostoc mesenteroides*, and *Latilactobacillus sakei* by 16S rRNA gene sequence analysis. *Leuconostoc mesenteroides* strains HIT231 and HIT252, and *Latilactobacillus sakei* strain HIT273 could grow at 5°C in MRS broth, but their optimum growth temperature was 20°C-30°C. These results suggest that psychrotrophic lactic acid bacteria presumed to be derived from rice *koji* are present in *godo*, which is one of the factors in the low-temperature ripening of *godo* in winter.**

Key Words: fermented soy foods; *godo*; lactic acid bacteria; psychrotroph

Introduction

Humans have traditionally made various fermented foods using the functions of microorganisms. Fermented foods are preserved foods that our ancestors made with their wisdom to survive, and they are also functional foods with enhanced nutritional value (Murooka and Yamshita, 2008; Ross et al., 2002). In Japan, there are a variety of fermented soybean products, for example, *miso* (soybean paste), *shoyu* (soy sauce), and *natto* (fermented soybean). For *miso* and *shoyu*, microorganisms such as *Aspergillus oryzae*, *Zygosaccharomyces rouxii*, and salt tolerant lactic acid bacteria, *Tetragenococcus halophilus* are involved in the fermentation process (Kim et al., 2010; Tanaka et al., 2012). *Natto*, on the other hand, is made of soybeans fermented by *Bacillus subtilis* (*natto*).

One such fermented food is called "*godo*" (Fig. 1). *Godo* is a traditional fermented food mainly made of soybeans, *koji*, and salt in winter. It used to be widely made in northeast Japan (Sugawara et al., 1995), but now it is only found in some districts of Towada City, Aomori Prefecture. The typical old manufacturing process of *godo* is as follows: 1. roast and peel soybeans; 2. add water to the soybeans and soak for 24 h; 3. steam or boil the soybeans until they are soft enough to be crushed; 4. wrap the soybeans in straw and keep warm in *kotatsu* (Japanese table with attached heater covered by blanket) for at least 24 h; 5. add cooked rice, rice *koji*, and salt water; 6. ferment at a low temperature for 20 days to 1 month (Aomoriken Nogyo Kairyo Fukyukai, 2005). The process varies by region and household (Fig. 2) and may also use barley, wheat, wheat bran *koji*, or soy sauce (Moriyama et al., 1986; Sugawara et al., 1995). The above steps 1 to 4 are the same as the process for making *natto*. In some cases, soybeans that did not string well when making *natto* in winter were diverted

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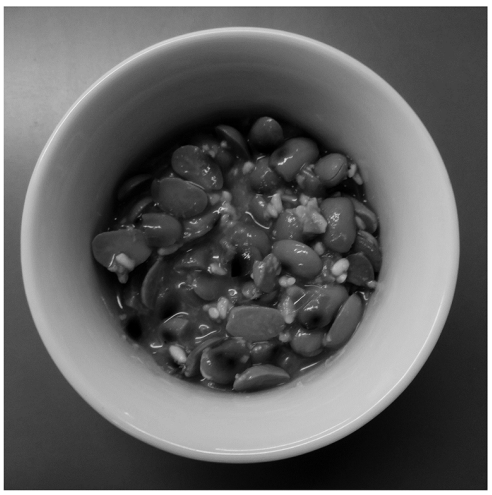

Fig. 1. Appearances of *godo*.

to *godo*.

Nowadays, due to social changes and the development of logistics, it is becoming difficult to eat unique cuisines in their home regions. The number of people who have inherited local dishes such as *godo* is decreasing, and its production method is not well known to the general public. Therefore, there is a risk of losing the microbial resources contained in *godo* as well as *godo* itself. Since *godo* has a sour taste, it is assumed that fermentation occurs by lactic acid bacteria. In recent years, it has been known that gut microbiota and their metabolites are closely related to the host health, and expectations for lactic acid bacteria in the maintenance of human health are increasing (Zommiti et al., 2020).

The purpose of the study was to investigate the presence or absence of psychrotrophic lactic acid bacteria in *godo*. Since *godo* ripened during the harsh winter in northeast Japan, the isolated strains were hypothesized to have cold tolerance. We isolated bacteria strains from *godo*, identified their species, and evaluated growth at a low temperature. The results revealed that the psychrotrophic strains of *Leuconostoc mesenteroides* and *Latilactobacillus sakei* were present in *godo*.

Materials and Methods

Determination of viable microbial count. Four homemade *godo* samples were obtained in Towada City. Materials and production process of each sample were shown in Fig. 2. All samples were brought to the laboratory in a cooler box and stored in the refrigerator. For microbial count, serial dilutions of *godo* samples in saline and four kinds of agar media were used. Standard plate count agar (SPC agar; Nissui, Tokyo, Japan) was incubated under aerobic condition at 35°C for 2 days for counting the total aerobic bacteria. The counts of lactic acid bacteria were measured by plate count agar with bromocresol purple (BCP agar; Nissui) with 10 ppm sodium azide and 10 ppm cycloheximide, and yellow colonies were counted after 2–3 days of incubation at 30°C. Gifu anaerobic medium (GAM agar; Nissui) was incubated under anaerobic condition at 35°C for 2 days for counting anaerobic bacteria. Potato dextrose agar (PD agar; Nissui) with 0.01% chloramphenicol was incubated under aerobic condition at 25°C for 5–7 days for counting fungi and yeast.

Biochemical analysis of metabolic products. Concentrations of lactic acid, acetic acid, and ethanol in *godo* samples were determined enzymatically using F-kit (Roche Diagnostics K.K., Tokyo, Japan) following the manufacturer's instructions.

Isolation of lactic acid bacteria strains and phenotypic characterization. From the incubated BCP agar plates, single yellow colonies indicating production of acids were randomly picked and purified by streaking. The pure isolates were cultured in Difco™ Lactobacilli MRS Broth (Difco Laboratories, Detroit, MI). The isolated strains were characterized by Gram staining and catalase test, and stored at -70°C in MRS broth containing 15% (v/v) glycerol until use.

Growth conditions of isolated strains. The strains were cultured in MRS broth for two passages (30°C, 24 h). After cultivation, the bacterial solution was inoculated 2% (v/v) into MRS broth and incubated at each temperature according to the experiment. Growth was determined by bacterial cell density (OD_{660}) and the culture medium pH during incubation.

Identification by 16S rRNA gene sequencing. The total genomic DNA of the strains HIT231, HIT252, HIT273, and HIT281 was extracted using achromopeptidase (FUJIFILM Wako Pure Chemical, Osaka, Japan), and the 16S rRNA gene was amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara Bio, Kusatsu, Japan). The 16S rRNA gene sequencing by dideoxy method were conducted using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA) and ABI PRISM 3500xL Genetic Analyzer (Applied Biosystems). Primers 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 515F (5'-GTGCCAGCAGCCGCGGT-3'), 1099F (5'-GCAACGAGCGCAACCC-3'), 536R (5'-GTATTACCGCGGCTGCTG-3'), 926R (5'-CCGTCAATTCCTTTGAGTTT-3'), and 1510R (5'-GGCTACCTTGTTACGA-3') were used for the gene sequencing (Nakagawa and Kawasaki, 2001). The obtained sequences of 16S rRNA gene were analyzed using NCBI-BLAST search program for the identification of the strains.

Carbohydrate fermentation analysis. The carbohydrate fermentation profiles were assessed using the API50 CH (BioMérieux, Marcy-l'Étoile, France) identification system according to the manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using R statistical software (version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria).

Results

Microbial populations

We collected four samples of *godo*. The results of microbial analysis are shown in Table 1. Aerobes and anaerobes containing lactic acid bacteria were detected in *godo*. On BCP agar, yellow colonies producing acid and purple colonies were detected from all the samples. The viable

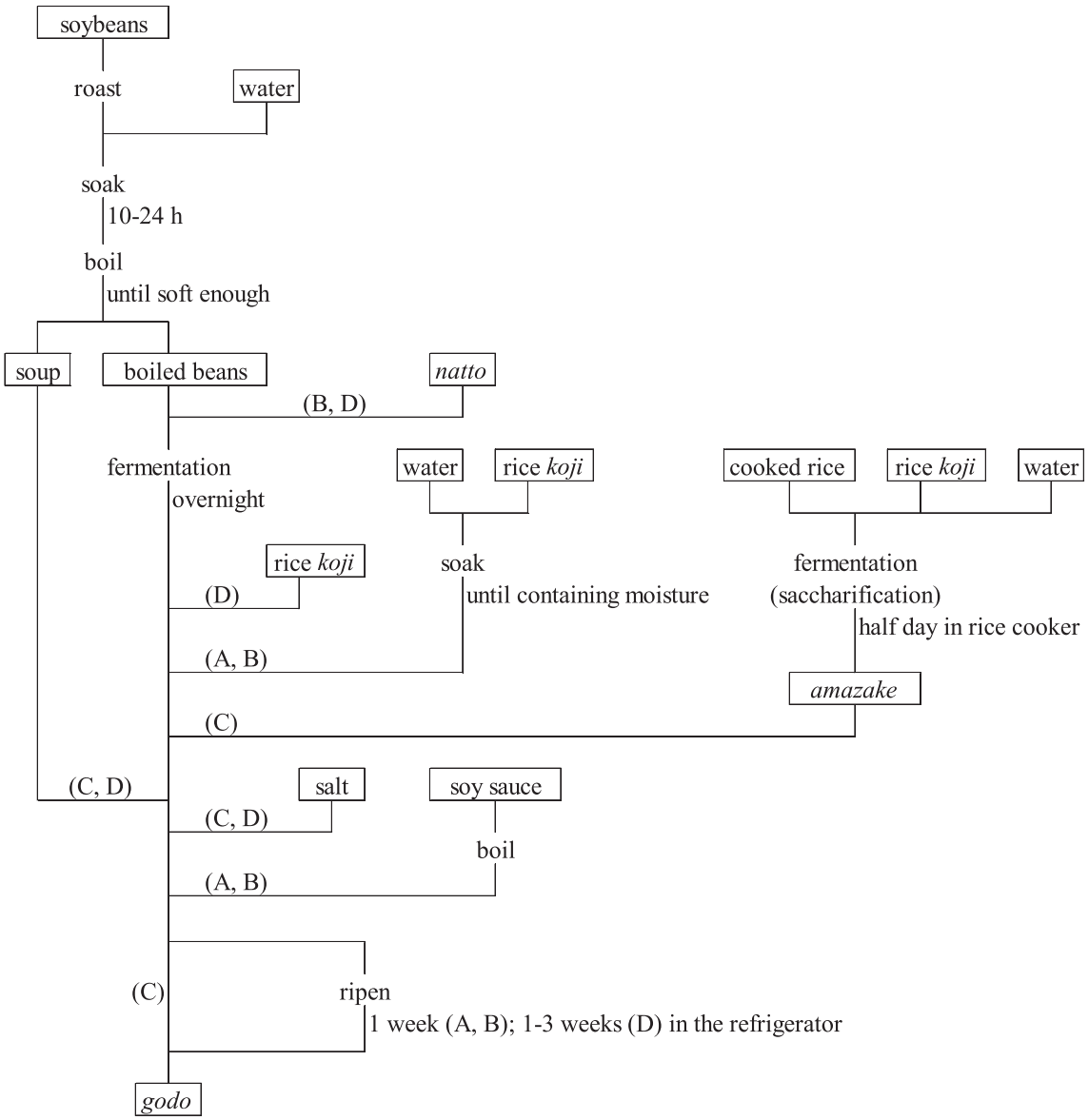

Fig. 2. Process outline of *godo*. Letters in parentheses indicate different *godo* process.

Table 1. Microbial counts of *godo* in culture media.

Sample	Microbial counts (Log cfu/g)			
	BCP	SPC	PD	GAM
A	6.72	4.43	4.40	8.34
B	7.04	7.59	ND	7.30
C	8.39	8.62	5.00	8.01
D	6.63	6.61	7.51	5.69

BCP, plate count agar with bromocresol purple with 10 ppm sodium azide and 10 ppm cycloheximide; SPC, standard plate count agar; PD, potato dextrose agar with 0.01% chloramphenicol; GAM, Gifu anaerobic medium; ND, not detected.

acid-producing bacteria ranged from 10^6 to 10^8 cfu/g. Molds and yeasts were not detected in sample B in PD agar. In sample A, anaerobic bacterial counts in GAM agar were 10 times more abundant than bacterial counts in BCP agar. These results indicated that the *godo* samples have a diverse microbial population.

Table 2. Contents of organic acids and ethanol in *godo*.

Sample	Lactate (mM)	Acetate (mM)	Ethanol (mM)
A	11.3	2.3	6.4
B	13.7	53.4	8.4
C	258.4	66.8	6.7
D	14.2	71.6	77.2

Metabolic products of fermentation

Lactate, acetate, and ethanol of *godo* samples were determined as products of fermentation. The metabolites differed depending on the sample (Table 2): sample C contained a large amount of lactate, while sample D had a higher ethanol concentration. Sample A and B tasted like soy sauce, and sample D had a sludgy, *miso*-like taste, while sample C had a strong sour taste.

Isolation of bacteria and phenotypic characterization

Twenty-two strains were obtained from four *godo* sam-

ples using BCP agar; nine strains from sample A and six strains from B, five strains from C, and two strains from D. Of the 22 strains, 16 were rod and 6 were cocci (Table 3). After 24 h cultivation, all the isolated strains grew in MRS broth and the pH values of media decreased to 4.14 ~ 4.60. All isolated strains were Gram-positive and catalase-negative (Table 3), which fits the classification of lactic acid bacteria.

Growth at 10°C

Since *godo* was fermented at low temperatures in winter, the cold tolerance of the lactic acid bacteria strains was evaluated. The optical density (OD_{660}) of the culture after incubation at 10°C was shown in Fig. 3. All isolates grew at 10°C in MRS broth. The strains HIT231, HIT252, and HIT273 showed the highest growth in each sample. They were selected as psychrotroph and candidate of psychrophile, and were used for the following experiment. Although the strains isolated from sample D did not show high growth at 10°C, strain HIT281 was selected for comparison with the above three strains.

Table 3. Taxonomic properties of lactic acid bacteria.

Strain	Cell shape	Gram stain	Catalase test
HIT225	Rod	+	–
HIT226	Rod	+	–
HIT227	Rod	+	–
HIT228	Rod	+	–
HIT229	Rod	+	–
HIT230	Cocci	+	–
HIT231	Cocci	+	–
HIT232	Rod	+	–
HIT233	Rod	+	–
HIT251	Rod	+	–
HIT252	Rod	+	–
HIT253	Rod	+	–
HIT254	Rod	+	–
HIT255	Rod	+	–
HIT256	Rod	+	–
HIT262	Cocci	+	–
HIT263	Rod	+	–
HIT264	Rod	+	–
HIT273	Rod	+	–
HIT276	Cocci	+	–
HIT280	Cocci	+	–
HIT281	Cocci	+	–

Carbohydrate fermentation analysis and identification by 16S rDNA sequencing

The API 50CHL analysis showed strains HIT231, HIT252, and HIT281 were able to utilize D-glucose and maltose contained in rice *koji*, but not starch (Table 4). The strains also utilize sucrose and raffinose contained in soybeans, while strain HIT273 lacked the ability to utilize maltose, raffinose, and starch.

Four strains with different isolation sources and patterns of carbohydrate fermentation were identified by 16S rRNA sequencing. As a result of BLAST homology search, strains HIT231 and HIT252 were identified as *Leuconostoc mesenteroides*; strain HIT273 was *Latilactobacillus sakei*; and strain HIT281 was *Enterococcus* sp.

Growth at 5°C and optimum temperature

To determine whether each isolate was psychrophilic or psychrotrophic, growth at 5°C and optimum temperature were evaluated. *Lt. sakei* HIT273 and *Leu. mesenteroides* HIT231 and HIT252 grew well at 5°C in MRS broth, albeit slowly (Fig. 4). *Enterococcus* sp. HIT281 showed slight growth at 5°C. The optimum temperature of growth was 30°C for *Lt. sakei* HIT273 and *Leu. mesenteroides* HIT231, 20°C for *Leu. mesenteroides* HIT252, and 37°C for *Enterococcus* sp. HIT281 (Fig. 5). These results demonstrated that the lactic acid bacteria strains in *godo* were not psychrophilic, but psychrotrophic.

Discussion

In this study, we investigated the lactic acid bacteria in *godo*. Viable cell count measurements revealed the pres-

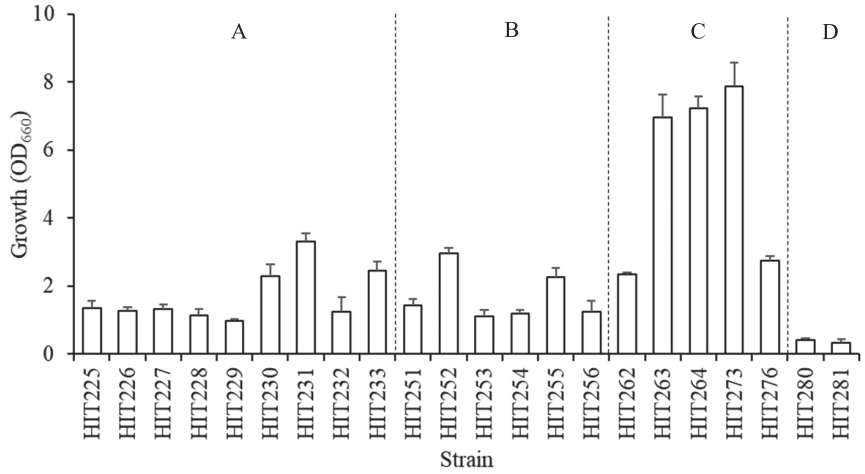

Fig. 3. Growth of the strains isolated from *godo* in MRS broth at 10°C.

Different letters indicate different samples. Data represent the mean values from three independent cultivations and standard deviation.

Table 4. Carbohydrate fermentation of isolated strains.

Strain	HIT231	HIT252	HIT273	HIT281
L-Arabinose	+	+	+	+
D-Ribose	+	+	+	+
D-Xylose	+	+	–	–
D-Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Rhamnose	–	–	–	–
D-Mannitol	+	+	–	–
N-Acetylglucosamine	+	+	+	+
Amygdalin	+	+	–	+
Salicin	+	+	–	+
Cellobiose	+	+	–	+
Maltose	+	+	–	+
Lactose	+	–	–	+
Melibiose	+	+	+	+
Sucrose	+	+	+	+
Raffinose	+	+	–	+
Starch	–	–	–	–

ence of various microorganisms. Three species classified as lactic acid bacteria were isolated from *godo*, and some strains showed significant growth at low temperature.

Natto and rice *koji*, sources of *A. oryzae* and *B. subtilis*, were used in *godo*, and several microorganisms including lactic acid bacteria were detected. The results implied that these microorganisms might have commensal interactions in *godo*. Previous studies have shown that *B. subtilis* (*natto*) enhances the growth and viability of lactobacilli, possibly by production of subtilisin, a serine protease (Hosoi et al., 2000). Zhang et al. (2014) pointed out that the consumption of oxygen by *B. subtilis* also had a positive effect on lactobacilli. From the results of API 50CHL (Table 4), the isolates cannot utilize starch, so they need other microorganisms to degrade starch to sugars. *A. oryzae*, derived from rice *koji*, produces amylase, which breaks down rice starch (Bechman et al., 2012). The availability of proteolytic products and sugars produced by *A. oryzae* and *B. subtilis* is considered to be nutritionally favorable for the lactic acid bacteria.

The strains classified as *Leu. mesenteroides*, *Lt. sakei*, and *Enterococcus* sp. were isolated from *godo*. *Leu. mesenteroides* and *Lt. sakei* were reported to be present in *kimoto*, which is the traditional seed mash made of rice *koji* used in Japanese sake production (Masuda et al., 2012; Takahashi et al., 2021). Enterococci has also been reported to be isolated from rice *koji* and *miso* (Ebine, 1985). The lactic acid bacteria isolated from *godo* seem to have originally come from rice *koji*.

The isolated strains of *Lt. sakei* and *Leu. mesenteroides* grew well at 5°C, and they had an optimum temperature of 20°C-30°C. This characteristic corresponds to a psychrotroph (Morita, 1975). Referring to previous reports, this cold tolerance is a general property of *Lt. sakei* (Torriani et al., 1996; Vermeiren et al., 2006) and *Leu. mesenteroides* (Hamasaki et al., 2003; Yamaner et al., 2010). Since *godo* ripened at low temperatures in winter, it is speculated that the cold tolerance of the strains favored their survival in *godo*.

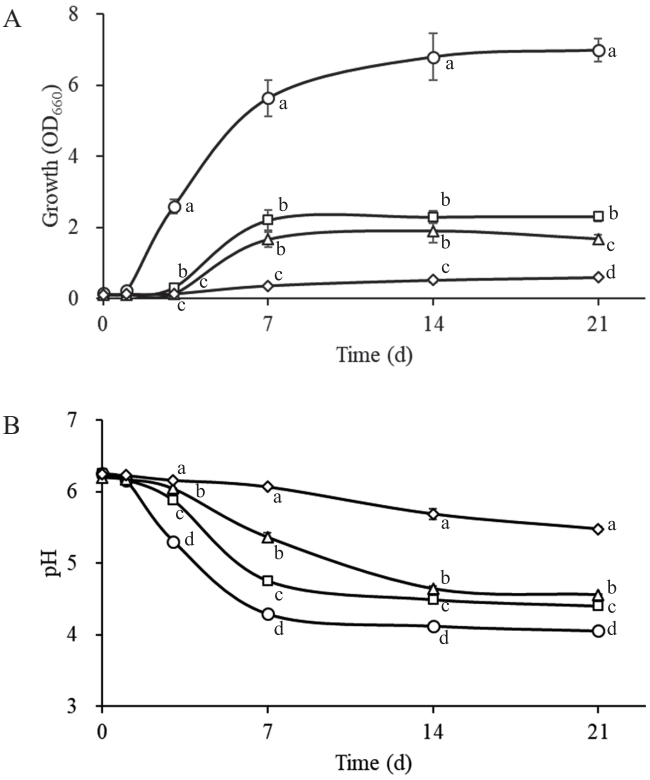

Fig. 4. Growth curve of four isolated strains from *godo* incubated in MRS broth at 5°C; (A) OD_{660}, (B) pH; □, HIT231; △, HIT252; ○, HIT273; ◇, HIT281.

Data represent the mean values from three independent cultivations and standard deviation. Different letters show a significant difference among the same culture time ($p < 0.05$, Games-Howell test).

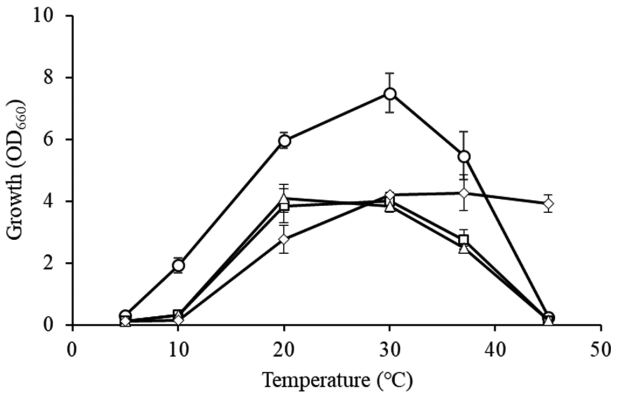

Fig. 5. Optimum temperature of growth in MRS broth; □, HIT231; △, HIT252; ○, HIT273; ◇, HIT281.

Data represent the mean values from three independent cultivations and standard deviation.

In sample B, no mold derived from rice *koji* was detected in the PD agar (Table 1). According to the interview with the producer of sample B, the sample was placed in a container with a deep bottom and fermented for about one week when it was made. The sample would have been anaerobic, and aerobic molds were reduced and not detected. While lactic acid fermentation occurred mainly in sample C, sample D contained a large amount of ethanol (Table 2), indicating that alcohol fermentation by yeast occurred. Sample A contained 10^8 cfu/g of anaerobic bac-

teria, suggesting that sample A contain more anaerobic bacteria than lactic acid bacteria. A wide variety of fungi and bacteria have been found in *miso* and rice *koji* (Allwood et al., 2021), which may also be true for *godo* as well. Because of the rich variation and diversity of microorganisms, *godo* can be a resource for a variety of microorganisms, not just lactic acid bacteria. As mentioned in the Introduction and Fig. 2, the method of making *godo* and the raw materials vary greatly from producer to producer. The psychrotrophic *Lt. sakei* was detected only in sample C, but the effect of different raw materials is unclear. There are only a few producers left, and fewer samples are available. It will be necessary to investigate the effects of raw materials and manufacturing processes on the microbial species.

In conclusion, the psychrotrophic lactic acid bacteria inhabiting *godo* were found to be *Lt. sakei*, and *Leu. mesenteroides*. Elucidation of the interaction of these lactic acid bacteria with *koji* mold and *natto* bacteria will contribute to the technological optimization for *godo* ripening process.

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