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Analysis of Lactic Acid Bacterial Flora during *Miso* Fermentation

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This article describes a microbiological study of lactic acid bacteria involved in the fermentation process of *Miso.* The bacteria were counted and isolated from *Miso* during fermentation and, based on the results of traditional phenotypic tests, divided into nine groups. The isolates were identified by biochemical analysis and 16S rRNA sequence analysis. During *Miso* fermentation, the halophilic bacterium *Tetragenococcus halophilus* increased moderately. The non-halophilic strains displayed a complex growth pattern and were identified as *Enterococcus faecium, Enterococcus durans, Enterococcus faecalis, Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum* and *Weissella confusa.* The predominant species throughout the fermentation process were *T. halophilus, E. faecium* and *E. durans.* Among them, only the strains of *E. faecalis* and *E. durans* produced bacteriocins that had an antibacterial effect on *B. subtilis*, but had none on *T. halophilus.* The bacteriocin producers appear to play an important role in maintaining normal bacterial flora during *Miso* fermentation.

Keywords: microflora, *Miso*, bacteriocin, fermentation lactic, acid bacteria, enterococci, *Enterococcus*

Miso (fermented soybean paste) is one of the traditional fermented soybean foods produced in Japan. It is used in the preparation of soups and other foods. Rice *Miso* (‘*Kome Miso*’) is made by grinding a mixture of cooked soybeans, rice *koji* (‘*Kome koji*’: a starter mold with cooked rice as the substrate) and salt, and fermenting the mixture in about 12% NaCl. In the past decade, several studies have been conducted on the physiological function of *Miso* (Ebine, 1990; Arifuku *et al*., 1993; Miyama *et al*., 1996; Hayashida *et al*., 1998; Yoshiki & Okubo, 1998). This focus has partly been due to a recent resurgence in the popularity of *Miso* and its processed products with the trend toward a healthier diet. There has also been a recent increase in the consumption of *Miso* outside Japan (Ebine, 1990).

The microbiology of *Miso* has been studied for a long time. The microflora of *Miso* commonly includes yeasts, molds and lactic acid bacteria. Among the lactic acid bacteria, the typical species (Yoshii, 1966, 1995, 1996; Ito & Warabe, 1994, Ohnishi & Tanaka, 1994; Okada, 1996) is highly halophilic *Tetragenococcus halophilus*, which is important in the fermentation and ripening processes of *Miso* production. In the latter stage of fermentation, during *Miso* ripening, halophilic *T. halophilus* becomes dominant, and is believed to be important in the acid production (decrease of pH), maintenance of the product’s bright color (the so-called *‘Sae’* effect), the induction of yeast growth, promotion of the desired flavor (the so-called *‘Shionare*’ and *‘Oshi-aji*’ effects) and the masking of offensive flavors (Yoshii, 1966, 1995, 1996; Ohnishi & Tanaka, 1994). In particular, the decrease of pH in *Miso* by lactic acid bacteria is important in inhibiting the growth of undesirable bacterial contaminants (Yoshii, 1995). The halotolerant *Enterococcus faecalis* strains are also present during the process of *koji*-making (Yoshii, 1966, 1996). Although these enterococci in *koji* are believed to be involved in acid production and maintenance of the product’s bright color, little is known about their behavior or role during rice *Miso* fermentation. Conversely, some lactic acid bacteria, including *Pediococcus acidilactici* (Yoshii, 1995, 1996), *Lactobacillus fructivorans* (Nikkuni *et al*., 1996) and *Lactobacillus plantarum* (Kato, 1999) are believed to impair the quality of *Miso* by causing over-acidification, the swelling of packaged *Miso* (the so-called *‘Fukure’* effect) and inhibiting the growth of *koji* mold as it is being made, respectively.

There is an increasing demand by consumers and the producers of *Miso*-processed foods for high quality *Miso* that is free of bacterial contaminants. Therefore, control of the conditions of lactic acid bacterial flora and the prevention of bacterial contaminants are necessary to improve the *Miso* quality. We recently reported that bacteriocin producing enterococci are widespread in *Miso* (Onda *et al*., 1999, 2002). The bacteriocin producers were identified as *E. faecium* and *E. durans*, which belong to the *E. faecium*-‘species group’, rather than *E. faecalis*, which was considered to be the dominant enterococcal species in *Miso*. It is reasonable to assume that the bacteriocin-producers associated with *Miso* may be important in preventing the growth of contaminants and maintaining normal microfloral conditions during the *Miso*-fermentation process. Bacteriocin-producing bacteria are beginning to be exploited in the manufacturing and preservation of *Miso* (Kato, 1999; Okada & Yanagida, 1999).

In order to utilize bacteriocin-producing lactic acid bacteria in *Miso* fermentation, it is necessary to understand the changes in microbial flora during the fermentation process. Although the microbial changes during *Miso* fermentation have been studied by Yoshii (1995) and Ito (1979), little information is available on

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lactic acid bacteria and their species-level evolution during the ripening of *Miso*. Information on these bacteria is even scarce at the strain level.

The aim of this study was to analyze the dynamics of the lactic acid bacterial community responsible for the fermentation process of *Miso*. We isolated and identified the lactic acid bacteria involved in this process and undertook time series analyses on the composition and behavior of bacteriocin-producing lactic acid bacteria during *Miso* fermentation.

Materials and Methods

The Miso samples An 80 kg sample of a typical rice *Miso* (*'Kome Miso'*), so-called *'Tanshokukei-ChuKaraKuchi-Miso* (a light colored and semi-salty tasting *Miso*)', was prepared. The rice *koji* was mixed well with cooked soybeans in a ratio of 1 : 2.9 by weight, which represented a 10 : 7 ratio of rice to soybean (raw materials) by weight. Sodium chloride and water were added to the mixture to give a final concentration of ca. 12% NaCl (actual measured value: 11.9%) and ca. 49% water (actual measured value: 49.4%). This preparation was made in a *Miso* plant located in Yamanashi prefecture, Japan. The *Miso* sample was placed in a sterilized tank, covered with a vinyl-sheet and then transferred to our laboratory. An 8 kg weight was put on top of the *Miso*-fermentation tank and sample was allowed to ferment for 15 weeks at 25°C. No starter culture of lactic acid bacteria nor yeasts was used in this study. The fermentation was spontaneous and uncontrolled, and consequently involved several food microorganisms.

Sampling and pH determination Sampling was performed eight times during fermentation: at 0, 1, 2, 4, 6, 8, 12 and 15 weeks from the onset of the process. At each sampling, 50 g of *Miso* was taken from the center of the fermentation tank (ca. 20 cm in depth) at different points. *Miso* samples were placed in a sterile stomacher bag and homogenized with 450 ml of sterile saline (0.9% NaCl) using a Stomacher (Lab-Blender 400, Seward, London, UK). Serial dilutions in saline were prepared to count lactic acid bacteria. The pH of a *Miso* slurry prepared from 10 g of *Miso* and 10 ml of sterilized-distilled water was determined.

Enumeration and isolation of lactic acid bacteria and yeast Appropriate serial dilutions of *Miso* samples in saline water were used for microbial enumerations of (non-halophilic) lactic acid bacteria and halophilic lactic acid bacteria with the following two media: (1) Man, Ragosa and Sharpe agar (MRS agar: Lactobacilli MRS broth; Difco Laboratories, Detroit, MI), adjusted to pH 7.0 for the estimation of non-halophilic strains and (2) NaCl-MRS agar (MRS media containing 10.0% sodium chloride), adjusted to pH 8.0 for halophilic strains; Onda *et al.*, 1999). The two agar media were used with 1.5% calcium carbonate ($CaCO_3$), 10 μg/ml sodium azide and 10 μg/ml cycloheximide (Uchimura & Okada, 1992a). The $CaCO_3$ acts as a non-diffusible buffer that localizes the acid producers. Sodium azide and cycloheximide prevent the growth of aerobic microorganisms.

All counts were done using a plate-counting assay. The portions of serially diluted samples in saline water were dropped into petri dishes and mixed with molten-media (45°C). The number of acid-producers forming a clear zone around the colonies was counted after incubation for 3–6 days at 30°C, using a plate with 30–300 colonies. To isolate and purify the isolates, 50–100 pinpoint colonies of putative lactic acid bacterial colonies were randomly selected at each sampling period, picked up and streaked on either a MRS or 10% NaCl-MRS agar plate. The isolates were examined for purity by observing their cellular morphology by phase-contrast microscopy. Plate cultures were incubated anaerobically using the anaerobic jar system (Rectangular Jar with Anaerobic Pack '*ANERO*'; Mitsubishi Gas Chemical Co., Inc., Tokyo).

Concurrent with the above, yeasts were counted using a PDA agar medium. The agar was supplied with 0.25% sodium propionate to prevent the growth of mold. The serially diluted samples were dropped, spread onto an agar-plate and incubated for 3–6 days at 25°C.

Evaluation of Miso sample *Miso* samples were analyzed for acidity (the so-called '*Sando* I' in *Miso* analysis), and for the contents of free amino nitrogen (formol nitrogen), L-lactic acid, acetic acid, L-glutamic acid and ethanol (by enzymatic methods), according to the standard method of *Miso* analysis (Yoshii *et al.*, 1996).

Bacterial cultures and cultural conditions All cultures of isolated strains were maintained as frozen stock (−80°C) in cultured broth, with 25% glycerol and 0.45% sodium chloride added for long-term storage. Working stab cultures (1.25% agar) were stored at 4°C. Isolated strains were propagated in MRS broth (pH 7.0) for non-halophilic strains and 6.5% NaCl-MRS broth (pH 8.0) for halophilic strains. The isolates were inoculated into the media and statically subcultured more than three times before assessing their antibacterial activity and biochemical characteristics. Agar plates were prepared with the addition of 1.5% agar to the broth medium, and the soft agar medium was prepared with 0.5% agar. The isolates were subcultured anaerobically. All media were autoclaved (121°C, 15 min) before use.

Morphological, physiological and biochemical characterization of lactic acid bacteria The isolated lactic acid bacteria from MRS agar and NaCl-MRS agar were subjected to characterization tests as described by Uchimura and Okada (1992b) and Sudo (1985). The isolates were characterized to genus level and divided into groups by the following traditional phenotypic tests: Gram reaction; cell morphology (cell shape and arrangement); catalase production; oxidase production; presence of spores; aerobic and anaerobic growth; gas production from glucose; growth at incubation temperatures of 10 and 45°C; growth at pH 9.6; growth in 6.5% NaCl; acid production from eight basal sugars (L-arabinose, D-ribose, D-xylose, D-fructose, mannose, lactose, sucrose and raffinose). The isolates that were Gram-positive, coccal or rod shaped, catalase-negative, oxidase-negative and non-spore-forming anaerobes, were considered to be lactic acid bacteria, and were subjected to further identification tests. Some of the isolates were Gram-positive and catalase-positive; these were assumed to be non-lactic acid bacteria and were omitted from further tests.

Identification of enterococci. Phenotypic tests, according to the test schemes of Facklam and Collins (1989) and Devriese *et al.* (1993), were conducted on selected putative enterococcal strains of homofermentative cocci that were grouped in chains (or occasionally in pairs) and had the ability to grow in the presence of 6.5% NaCl, at pH 9.6 and at 15 and 45°C. The tests were: growth at 50°C; Voges-Proskaner reaction; bile-esculin reaction; ammonia production from arginine; pyrrolidonylaryla-

midase reaction; hydolysis of hippurate; α-galactosidase reaction; pseudocatalase production; reduction of 0.05% potassium tellurite and 0.01% tetrazorium blue; decarboxylation of arginine and tyrosine (Møller, 1955); pigment production; motility; hemolysis; configuration of lactic acid (Okada *et al*., 1978); acid production using the 29 carbon sources, including the above 8 sugars, listed in Table 1. The test for group D-antigen by agglutination was done using a D-antigen kit (Sigma Chemical Co., MS). Drug (antibiotic) resistance was tested using the four antibiotic discs (tri-discs *'EIKEN'*: vancomycin, ampicillin, erythromycin and tetracycline, Eiken Co., Tokyo), which were applied to tested bacterial lawns on MRS-agar plates. Proteolytic activity was investigated using the skim milk agar test by Lu *et al*. (1994). Autolytic activity in the cultured broth was investigated by measuring the change in absorbance at 660 nm.

Identification of tetragenococci and pediococci. Phenotypic

Table 1. Morphological, physiological and biochemical properties of the lactic acid bacteria isolated from *Miso*.

Test		Divided group[a)]									
		I	II	III	IV	V	VI	VII	VIII	IX	X
No. of isolated strains		101	246	128	179	54	40	43	22	39	9
(selective strains for identification)		(7)	(35)	(28)	(10)	(4)	(3)	(2)	(2)	(2)	(2)
Cell morphology		cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	rod	rod
Cell arrangement		tetrad	chained	chained	chained	chained	tetrad	tetrad	tetrad	−	−
Gas production from glucose		−	−	−	−	−	−	−	−	−	+
Growth:	at 10°C	W	W	W	−	W	−	−	W	−	−
(tolerant)	at 15°C	W	W	W	W	W	W	W	W	W	W
	at 40°C	+	+	+	+	+	+	+	+	+	+
	at 45°C	+	+	+	+	+	+	+	+	+	+
	at 50°C[b)]	NT	NT	NT	NT	NT	+	W	−	W	NT
	in 6.5% NaCl	+	+	+	+	+	+	+	+	+	+
	in 10.0% NaCl	+	−	−	−	−	−	−	−	−	−
	in pH 4.2	−	−	−	−	−	+	+	+	NT	NT
	in pH 8.5[b)]	+	+	+	+	+	+	+	+	NT	NT
	in pH 9.6[b)]	+	+	+	+	+	+	W	−	−	−
Optical form of lactic acid		L(+)	L(+)	L(+)	L(+)	L(+)	DL	DL	DL	DL	DL
Acid from[c)]:	L-Arabinose	+	W/+	+	−	−	−	−	−	+	−
	Glycerol	W	−	−	−	+	−	−	−	−	−
	Gluconate	−	−	−	−	+	−	−	−	+	−
	Lactose	−	+	+	W	+	−	−	+	+	−
	Maltose	+	+	+	+	+	−	+	+	+	+
	Mannitol	−	W/−	+	+	W/+	−	−	−	+	−
	D-Mannose	+	+	+	+	+	+	W	+	+	−
	Melezitose	+	−	−	−	+	−	−	−	+	−
	Melibiose	−	+	+	+	+	−	−	−	+	−
	Raffinose	−	W/+	+	+	W/+	−	+	+	−	−
	L-Rhamnose	−	+	+	+	+	−	−	−	−	−
	Sorbitol	−	−	−	−	+	−	−	−	+	−
	Sucrose	+	+	+	+	+	−	−	+	+	W/+
	D-Tagatose	−	−	−	−	+	−	−	−	−	−
	Trehalose	+	W/−	W/+	+	+	W/+	−	+	−	+
	D-Xylose	−	W/+	W/−	−	−	W/−	W	−	−	W/+
Voges-Proskauer reaction[d)]		NT	+	+	+	+	NT	NT	NT	NT	NT
Bile-esculin reaction[d)]		NT	+	+	+	+	NT	NT	NT	NT	NT
D-Antigen agglutination[d,e)]		NT	+	+	+	+	NT	NT	NT	NT	NT
Production of:											
Pseudocatalase		−	−	−	−	−	−	+	−	−	−
Arginine hydrolase[d)]		NT	+	+	+	+	NT	NT	NT	NT	NT
Hippurate hydrolase[d)]		NT	+	+	+	+	NT	NT	NT	NT	NT
Pyrrolidonylaryamidase[d)]		NT	+	+	+	+	NT	NT	NT	NT	NT
α-Galactosidase[d)]		NT	−	−	−	−	NT	NT	NT	NT	NT
Decarboxylation[d)] of:											
Arginine		NT	+	+	+	+	NT	NT	NT	NT	NT
Tyrosine		NT	+	+	+	+	NT	NT	NT	NT	NT
Growth[d)] in:	0.25% tetrazolium	NT	−	−	−	+	NT	NT	NT	NT	NT
	0.04% tellurite	NT	−	−	−	+	NT	NT	NT	NT	NT
Yellow pigment production[d)]		NT	−	−	−	−	NT	NT	NT	NT	NT
Vancomycin-resistant[d,f)]:		NT	S	S	S	S	NT	NT	NT	NT	NT
Haemolysis[d,g)]		NT	−	−	−	−	NT	NT	NT	NT	NT
Proteolytic activity[d,h)]		NT	−	−	−	+	NT	NT	NT	NT	NT
Autolytic activity[d,i)]		NT	−	−	−	+	NT	NT	NT	NT	NT
DAP in cell wall[j)]		NT	NT	NT	NT	NT	NT	NT	NT	+	−
Bacteriocin activity[k)]		+	−	−	+	−	−	−	−	−	−

+: positive reaction; W: weakly positive reaction; —: negative reaction; s: sensitive.
[a)] Strains of group I were isolated on 10%NaCl-MRS agar and strains of groups II–X were isolated on MRS agar. All strains were Gram-positive, catalase-negative, oxidase-negative, non-spore-forming and non-motile. [b)] Test for putative pediococci. [c)] All strains produced acid from glucose, arbutin, cellobiose, galactose, D-fructose, D-ribose and salicin; the acid was not produced from erythritol, D-fucose, glycogen, inulin, 2-keto-gluconate or L-sorbose. [d)] Test for putative enterococci. [e)] Test using a D-antigen agglutination kit. [f)] All strains were also sensitive to ampicillin, erythromycin and tetracycline. [g)] Test using sheep-blood agar. [h)] Test on skim milk agar. [i)] The autolytic activity in the cultured broth was determined by measuring the decrease in absorbance at 660 nm. [j)] Test for putative lactobacilli. [k)] +: bacteriocin activity positive in 18 h-cultured broth; −: no activity.

Table 2. Comparison of antibacterial activity and its spectra of bacteriocin-producing enterococci.

The bacteriocin-producer (upper) and *Miso*-related strain (lower)		The bacteriocin and its antibacterial activity (AU/ml)[a] of				
		E. durans MF001	*E. durans* MF101	*E. durans* MF1201	*E. faecalis* MF404	*E. faecalis* MF405
		80	160	1280	320	320
E. durans	MFL001	−	−	+++	−	++
	MFL101	++	−	+++	−	−
	MFL1201	−	−	−	−	−
E. faecalis	MFL404	++	−	+++	−	++
	MFL405	++	+	+++	++	−
P. acidilactici	JCM5885^T	+	+	+++	++	++
	MFL011	+	+	+++	++	++
L. plantarum	JCM1149^T	++	−	+++	++	++
	MFL401	++	−	+++	++	++
L. fructivorans	JCM1117^T	−	−	++	−	−
		−	−		−	−
W. confusa	MFL014	−	−	+++	−	−
		−	−	−	−	−
T. halophilus	JCM5888^T	−	−	−	−	−
	MLHL001	−	−	−	−	−
B. subtilis	JCM1465^T	++	+	++	+	++

+++: highly sensitive (large [>8 mm diameter] inhibition zone on agar well diffusion method), ++ sensitive, +: weakly sensitive, −: insensitive. JCM: Japan Culture Collection of Microorganisms, RIKEN (Saitama).

[a] Bacteriocin activities (AU/ml) in 18 h-cultured MRS broth were measured by agar-well diffusion method using *Lactobacillus sakei* JCM1157^T as an indicator strain.

tests, according to the test scheme of Uchimura and Okada (1992b), were conducted on selected putative pediococcal strains, which were homofermentative cocci grouped in tetrads (or in pairs). The tests were: growth at 40 and 50°C; growth at 5, 10 and 12.5% NaCl; growth at pH 4.2 and 8.5; acid production using the 29 carbon sources listed in Table 1 (including the above 8 sugars); pseudocatalase production; motility and configuration of lactic acid.

Identification of lactobacilli. Phenotypic tests, according to the test scheme of Uchimura and Okada (1992b), were conducted on selected putative lactobacilli strains that had rod-shaped cells. The tests were: growth at 15 and 50°C; acid production using the 29 carbon sources listed in Table 1 (including the above 8 sugars); presence of *meso*-diaminopimelic acid (DAP) in cell-peptidoglycan; motility and configuration of lactic acid.

Chromosomal DNA preparation and 16S rRNA sequence analysis Chromosomal DNAs were prepared from ca. 2 g of wet bacterial cells cultured overnight in MRS medium at 30°C, according to the method of Saito and Miura (1963), with some modifications. The prepared DNA had A_{235}/A_{260} and A_{280}/A_{260} ratios of less than 0.55. PCR amplification of the 16S rRNA gene from chromosomal DNA and the sequencing analysis of PCR products were carried out according to the method of Shida *et al.* (1997). Previously published 16S rRNA sequences were obtained from the database of the EMBL-GenBank-DDBL, available at the internet site http: //www.ddbj.nig.ac.jp/.

Detection and measurement of antibacterial activity The production of antibacterial substances by the isolates from *Miso* was confirmed by the detection of antibacterial activity, using the agar-well diffusion method (Tagg & McGiven, 1971). *Lactobacillus sakei* (Trüper & De'Clari, 1997) JCM1157^T, which showed high sensitivity (Onda *et al.*, 1999) against the bacteriocin produced by strain GM005, was used as the indicator. Inhibition of the indicator culture was detected by a clear zone around the test well, and the positive strain was confirmed for the absence of bacteriophages, using the modified method of the agar-well diffusion assay of Onda *et al.* (1999). An adoption of the critical dilution assay was used for the titration of antibacterial activity. The antibacterial titers were defined as the reciprocal of the highest dilution exhibiting a complete inhibition zone on the indicator lawn, and were expressed as antibacterial activity units (A.U.) per milliliter.

Effects of hydrolytic enzymes, detergents and heat treatment on antibacterial activity The effects of the hydrolytic enzymes, pronase, dispase, trypsin, α-chymotrypsin, cellulase, catalase (Roche Diagnostics GmbH, Mannheim, Germany), proteinase K (Sigma Chemical) and α-amylase (Wako Pure Chemical, Osaka) on the partially purified bacteriocin were determined by the method described by Onda *et al.* (1999).

Antibacterial spectra of bacteriocin Antibacterial spectra were investigated using the selected indicator strains related to *Miso* fermentation, as shown in Table 2.

Results

Changes in viable cell counts of lactic acid bacteria during Miso fermentation Changes in the counts of the two lactic acid bacterial groups (halophilic and non-halophilic groups), yeasts and the pH values measured during *Miso* fermentation, are presented in Fig. 1. The halophilic lactic acid bacterial counts were low (10^3 CFU/g) before ripening and increased moderately to 10^6 CFU/g between four and 15 weeks of fermentation. The yeasts also increased moderately to 10^4 CFU/g between five and 15 weeks. Conversely, the changes in non-halophilic lactic acid bacterial counts displayed a complex pattern: they increased moderately for the first week of fermentation, decreased steeply until week four, increased sharply to week six and thereafter decreased moderately to week 15. This pattern was similar to the observations of Ito (1979) and Yoshii (1988). The complex change appears to have been due to the evolution of two or more kinds of dominant lactic acid bacterial species. The growth patterns of the two bacterial groups during *Miso* fermentation were confirmed in three independent fermentation steps (Fig. 1). The *Miso* fermented for 12 weeks contained 0.45% free amino nitrogen, an 8.5 value of '*Sando* I' acidity, 0.25% L-lactic acid, 0.04%

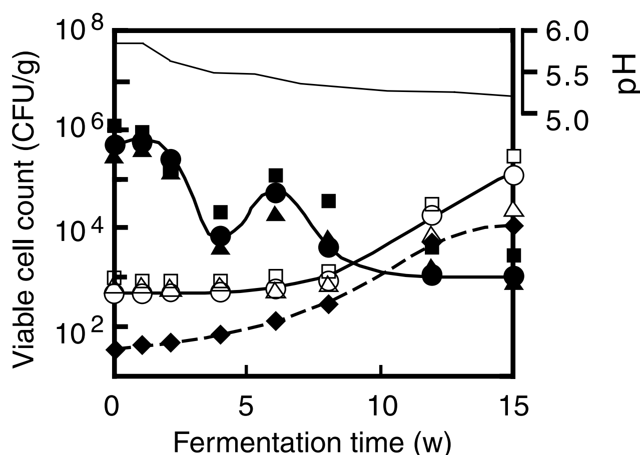

Fig. 1. Changes in viable cell-counts of halophilic (○), non-halophilic lactic acid bacteria (●) and yeasts (◆) including pH (line), during the *Miso*-fermentation process. Cell counts of halophilic (□ and △) and non-halophilic (■ and ▲) lactic acid bacteria obtained in two other independent experiments are also shown.

acetic acid, 0.31% L-glutamic acid and 0.51% ethanol. The *Miso* obtained in this study was evaluated as 'normal grade' (a satisfactory product) from the analyses done.

On the other hand, the number of aerobic mesophilic bacteria was constant (ca. 10^5 CFU/g) throughout the fermentation process (data not shown), which is in agreement with previous reports on the microbiology of *Miso* (Yoshii, 1988, 1995). The analysis of aerobic bacterial flora in *Miso* will be reported in our next paper.

Identification of lactic acid bacterial isolates One hundred and one lactic acid bacterial strains were isolated from 10% NaCl-MRS agar and 760 were isolated from MRS agar, resulting in a total of 861 strains from the *Miso* samples taken at various times during the fermentation process. These strains were confirmed to be lactic acid bacteria because they were Gram-positive, catalase and oxidase negative, non-spore-forming and they displayed lactate-fermentative catabolism. The exceptional isolates, which were Gram-positive and catalase-positive, were assumed to be non-lactic acid bacteria and were not tested further. All isolates were divided into the halophilic group (group I: 101 strains isolated from 10%NaCl-MRS agar) or the non-halophilic groups (groups II-XI: 760 strains isolated from MRS agar) by traditional phenotypic tests. The representative strains from the nine groups were characterized and identified by further phenotypic and biochemical tests. Their morphological, physiological and biochemical properties are shown in Table 1. The identifications were confirmed by 16S rRNA sequencing analysis of some strains. The identification of the groups were as follows.

Group I: Tetragenococcus halophilus (101 strains). All halophilic strains isolated from NaCl-MRS agar showed almost identical phenotypes, with round cells arranged in tetrads (or occasionally in pairs), the ability to grow in the presence of 10 and 12.5% NaCl and having halophilic characters. The seven selected strains grew at pH 8.5 but not at pH 4.2, indicating that they belonged to the species *T. halophilus*. The representative strains MYHL001 and MYHL1202 were also identified as *T. halophilus* by 16S rRNA sequencing analysis. *T. halophilus* is a typical lactic acid bacterium present during *Miso* fermentation.

Groups II, III, IV and V: Genus Enterococcus (607 strains; 80% of the 760 non-halophilic strains). All strains in groups II-VI grew in the presence of 6.5% NaCl, at pH 9.6, and at 15 and 45°C. These strains were classified into genus *Enterococcus* Schleifer and Kilpper-Bälz (1984).

Groups II, III and IV (553 strains; 73%). The strains of these groups were identified as strains of the *Enterococcus faecium*-'species group' (*E. faecium, E. durans* or *E. hirae*), which are known to be widespread in *Miso* products (Onda *et al.*, 2002). Groups II-IV were divided according to their carbohydrate-fermentation patterns, and the selected strains were differentiated from other known enterococcal species by some of their taxonomic properties. As their phenotypic characters were highly diverse (Table 1), these strains could not be identified to species level on the basis of their phenotypes (Facklam & Collins, 1989; Devriese *et al.*, 1993). However, they were identified by 16S rRNA sequencing analysis as follows.

Group II: E. durans (246 strains; 32%). Representative strains: MYL001, MYL101, MYL804 and MYL1201 were identified as *E. durans*. Group III: *E. durans* (128 strains; [17%]). Representative strains: MYL110, MYL212, MYL409 and MYL1202 were also identified as *E. durans*.

Group IV: E. faecium (179 strains; 24%). Representative strains MYL211 and MYL816 were identified as *E. faecium*.

Group V: E. faecalis (54 strains; 7%). The strains of this group were assigned to *E. faecalis*, using the identification schemes of Facklam and Collins (1989) and Devriese *et al.* (1993). Identification was based on their ability to grow in the presence of tetrazorium and tellurite, and on their carbohydrate-fermentation pattern. Representative strains: MYL404, MYL405 and MYL406 were confirmed to be *E. faecalis* by 16S rRNA sequencing analysis. The strains of *E. faecalis* showed relatively high proteinase activity on skim milk agar and had strong autolytic activity in the stationary phase.

Groups VI, VII, VIII: Genus Pediococcus (105 strains; 14%). The strains of these groups were arranged in tetrads (or occasionally in pairs). Group VI: *P. acidilactici*. Forty strains (5%) were identified as *P. acidilactici* by phenotypic characters, due to their growth at pH 8.5, pH 4.2 and at 50°C, and due to their carbohydrate-fermentation pattern. Representative strains: MYL011 and MYL114 were confirmed to be *P. acidilactici* by 16S rRNA sequencing analysis. This species was previously shown to be related to the cause of over-acidification in *Miso* (Yoshii, 1995). Groups VII (43 strains; 6%) and VIII (22 strains; 3%): *P. pentosaceus*. The strains were identified as *P. pentosaceus* by phenotypic characters, due to their growth at pH 8.5 and pH 4.2, their absence of or weak growth at 50°C, and their carbohydrate-fermentation patterns. The Group VII strains only produced pseudocatalase. Representative strains: MYL103, MYL202, MYL401 (group VII) and MYL211 (group VIII) were confirmed to be *P. pentosaceus* by 16S rRNA sequencing analysis.

Group IX: Lactobacillus plantarum (39 strains; 5%). The strains were homofermentative, rod-shaped, produced DL-lactate and had *meso*-DAP in their cell walls. The strains were identified as *L. plantarum* or *L. pentosus* by phenotypic characters, and by using their carbohydrate-fermentation patterns as an indicator of their ability to ferment arabinose and gluconate. Representative strains: MYL102 and MYL204 were confirmed to be *L. plantarum* by 16S rRNA analysis. This species appears to inhibit the

growth of *koji* (Kato, 1999).

Group X: Weissella confusa (9 strains; 1%). These strains were the only hetero-fermentative lactic acid bacteria isolated in this study. They were short, rod-shaped and had no *meso*-DAP in their cell walls. The group was identified as *W. confusa* by its phenotypic characters and carbohydrate-fermentation patterns. Representative strain: MYL014 was confirmed to be *W. confusa* by 16S rRNA analysis. Hetero-fermentative *L. fructivorans* was previously believed to be the cause of *'Fukure'* (swelling) during *Miso* fermentation (Nikkuni *et al.*, 1996), which results in the swelling of packaged *Miso* or *Miso*-processed products. The isolates of *W. confusa* in this study produced a large amount of gas from sugar in broth culture, suggesting that it is also a cause of *'Fukure'*. This is the first report on the isolation of *W. confusa* strains from *Miso*.

All non-halophilic strains: groups II-X were moderately halotolerant, as evidenced by their ability to grow in 6.5% NaCl.

Detection and comparison of bacteriocins produced by the isolated lactic acid bacteria All of the isolated lactic acid bacteria were examined for antibacterial activity. Only the strains of *E. durans* (group II) and *E. faecalis* (group V) produced antibacterial substances. These substances were sensitive to proteinaceous enzymes but were not affected by α-amylase, cellulase or catalase (data not shown). The antibacterial activity of the isolates was not affected by catalase treatment, indicating that these activities were not the action of hydrogen peroxide. This result suggests that the antibacterial substances were bacteriocins, which are proteinaceous substances.

The bacteriocins produced by the isolated bacteriocin-producers were grouped into five categories, based on the patterns of their antibacterial spectra. The antibacterial activity and antibacterial spectra of five representative bacteriocin producers against the selected bacteria, which are related to *Miso* fermentation, are summarized in Table 2. Among *E. durans* of group II, the isolates (representative strains: MYL001 and MYL101) from the early stage of *Miso* fermentation displayed relatively weak antibacterial activity (Table 2). Conversely, isolates (representative strain: MY1201) from the latter stage displayed the strongest antibacterial activity and its bacteriocin had the broadest spectrum (Table 2). The bacteriocin of *E. durans* MY1201 had an antibacterial effect on undesirable bacteria such as: *B. subtilis* JCM1465^T; *P. acidilactici* JCM5885^T, IAM1233, MYL011; *L. fructivorans* JCM1117^T, IFO3954; *L. plantarum* JCM1149^T, MYL204 and *W. confusa* MYL014. However, the bacteriocins of these isolates had no antibacterial effect on *T. halophilus* JCM5888^T or MLHL001, which are useful bacterial species in *Miso*.

Evolution of lactic acid bacterial flora during Miso-fermentation The quantitative evolution of halophilic and non-halophilic lactic acid bacterial microflora was analyzed during the fermentation of *Miso*. The halophilic group consisted of only *T. halophilus* throughout fermentation. Conversely, the proportion of the non-halophilic groups showed complex changing patterns during fermentation. Figure 2 shows the time-series changes in the proportion (%) of each of the species groups II–X. Most species isolated in this study were present from the onset of fermentation until week four. The strains of *P. acidilactici, P. pentosaceus, L. plantarum* and *W. confusa* disappeared between weeks two and 12. The composition of the flora changed drastically between four and eight weeks from the onset of fermentation; during this time, the most dominant species was *E. faecalis*. The proportion of *E. faecalis* rose sharply during the first four weeks of fermentation and then decreased sharply from weeks six to 12. At the end of 12 weeks, when fermentation was complete, strains of *E. durans* (groups II and III) and *E. faecium* (group IV) were the only microflora present. Among the isolates of *E. durans* (groups II and III) and *E. faecium* (group IV), the bacteriocin-producing strains of *E. durans* (group II) increased in their proportion during the latter stage of fermentation. Conversely, the proportion of non-bacteriocin-producing strains of *E. durans* (group III) and *E. faecium* (group IV) decreased.

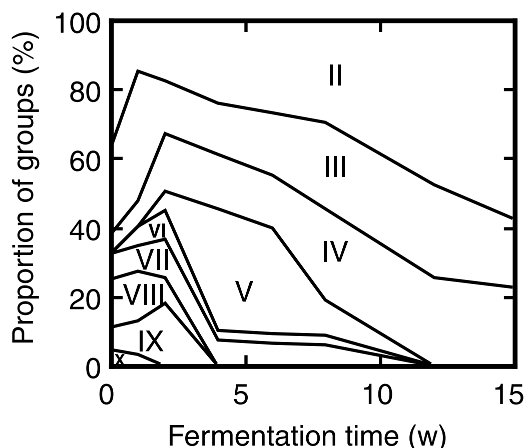

Fig. 2. Changes in the proportion of non-halophilic lactic acid bacterial groups during the *Miso*-fermentation process. II: *Enterococcus durans*; III: *E. durans*; IV: *E. faecium*; V: *E. faecalis*; VI: *Pediococcus acidilactici*; VII: *P. pentosaceus*; VIII: *P. pentosaceus*; IX: *Lactobacillus plantarum*; X: *Weissella confusa*. Most strains of *E. durans* (group II) and *E. faecalis* (group V) produced bacteriocins.

Discussion

The results of chemical and physical analyses of the *Miso* sample indicate that *Miso* fermentation by lactic acid bacteria and yeasts occurred normally. A well-known factory was chosen because its manufacturing and quality of *Miso* are relatively stable all year around.

The non-halophilic strains, mostly enterococci, showed complex growth patterns during fermentation (Fig. 1). In *Tanshokukei-ChuKaraKuchi-Miso* making, Ito and Warabe (1994) reported that non-halophilic strains (enterococci) decreased from the onset of *Miso* fermentation, then increased showing a single peak, and decreased again. According to them, this change in microflora was due to adaptation to salty conditions. In this study, the complex changes can also probably be attributed to changes in the dominant species of lactic acid bacteria.

Many lactic acid bacterial species were present during the early stage of *Miso* fermentation. During the early stage of normal fermentation, some undesirable species, such as *P. acidilactici, L. plantarum* and *W. confusa*, were present but they disappeared between weeks four and 12. If the undesirable strains become dominant, the quality of the *Miso* product will immediately be spoiled. The local dip in the total cell counts at week four of fermentation was most likely due to the disappearance of these undesirable strains. The composition of flora drastically

changed around week four, when there was a sharp increase in the abundance of enterococcal strains. The increase in total cell counts between weeks four and six of fermentation is probably due to an increase in the diversity and abundance of these strains. It is important and interesting to note the possible growth of enterococci in *Miso*. We will study the prevalence and dynamics of enterococci during *Miso*-fermentation in the future. After four weeks of fermentation, the strains of *E. faecalis* decreased rapidly until their complete disappearance by week 12. The sharp decrease of this species could have been due to autolysis, which might have been induced by alcohol produced by the yeasts. Indeed, the growth of lactic acid bacteria was considered to be influenced by growth of yeasts and/or other micro-organisms. The interaction between these microorganisms is important for understanding the formation of the *Miso* microflora, and this research will be conducted at a later date.

The dominant lactic acid bacteria in the latter stage of fermentation and in the final *Miso* product were halophilic *T. halophilus* and the non-halophilic (moderately halo-tolerant) strains of the *E. faecium*-'species group'. We recently reported that these comprised the dominant lactic acid bacteria in non-sterilized *Miso* products (Onda *et al*., 2002), which is in agreement with the findings of the present study. The halophilic and/or halo-tolerant characteristic could be a dominant factor, which would explain why these species were present throughout the entire fermentation process. On the other hand, the bacteriocin-producing strains of *E. durans* comprised the predominant microflora in the latter stage of fermentation. Among the bacteriocin-producing strains of *E. durans* (group II), the isolates in the latter stage had stronger antibacterial activity than those in the early stage. This observation suggests that the production of bacteriocins may be one of the dominant factors of lactic acid bacteria in *Miso* fermentation.

We recently reported that the enterococcal strains isolated from *Miso* are non-haemolytic and sensitive to antibiotics (vancomycin) and therefore, presumably non-pathogenic (Onda *et al*., 2002). In the present study, the isolated enterococci were also non-pathogenic (Table 1).

Although a large number of studies have been made on *T. halophilus* involving *Miso* (Ohnishi & Tanaka, 1994), little is known about the role of enterococcal bacteria in *Miso*. The bacteriocins produced by enterococcal isolates showed antibacterial activity to the undesirable bacteria in *Miso*: *B. subtilis, P. acidilactici* and *L. fructivorans*, but they did not inhibit the growth of the useful bacteria, *T. halophilus*. Our study suggests that the bacteriocin-producing enterococci are very important in preventing the growth of undesirable bacteria and maintaining the normal bacterial flora, so it is reasonable to assume that the bacteriocin producers associated with *Miso* may be important for improving the quality of *Miso* products.

The prevention of bacterial spoilage is becoming a major concern for producers of *Miso*. The bacteriocin of *E. durans* might be useful as a safe food bio-preservative to control spoilage and prevent undesirable bacterial growth, especially during *Miso*-fermentation. It is also possible that naturally occurring *E. durans* is a key microorganism for *Miso*-fermentation. Using a bacteriocin-producer as the starter microorganism could promote a more stable *Miso*-fermentation process and increase the reliable production of an excellent *Miso*-product.

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Appendix

Tetragenococcus halophilus was reclassified from *Pediococcus halophilus* based on a DNA-DNA hybridization study (Collins *et al*., 1990). *Enterococcus faecalis* and *Enterococcus faecium* were species reclassified from *Streptococcus faecalis* and *Streptococcus faecium*, respectively, based on a 16S rRNA sequencing study (Schleifer & Kilpper-Bälz, 1984). *Lactobacillus sakei* was renamed from *Lactobacillus sake*, according to Rule 12c of the International Code of Nomenclature of Bacteria (Trüper & De'Clari, 1997).