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Effects of Inoculation of Lactic Acid Bacteria Starter and Addition of Organic Acid on "NISHINZUKE" Fermentation

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Introduction

"NISHINZUKE" has been popular for many years as a preservable food in homes in Hokkaido during the winter months. NISHINZUKE is made from a mixture of herring and other animal products and KOJI (malted rice), and its low-salt fermentation makes it popular not only for its taste, but also for its nutritional value. However, in recent years, there has been a continuous trend toward a lower salt content in pickled foods due to changes in the diet of Japanese and the fact that Japanese have become more health-conscious. This has given rise to problems in maintaining the quality of NISHINZUKE in the distribution stage. Thus, it has become important to examine, not only the traditional production methods, but also the fermentation mechanism for a higher quality product. However, to date, there has been no detailed scientific investigation of pickling, such as of ingredients and regional differences, especially regarding vinegar pickling in Western countries[10], which is a form of preservation in which bacterial growth is retarded. However, in low-salt pickles, the salt inhibits the fermentative action of the vegetables and causes intracellular matter to elute. The fermentation process is due to bacteria, especially lactic acid bacteria, and preservation is enhanced by using this antagonism.

Various studies on pickling processes have been carried out in the past. Ogawa *et al.*[15] examined a scientific investigation of pickling methods, the effect of osmotic pressure on the quality of pickles during fermentation. Miyao *et al.*[12,13] studied the ecology of bacteria in the fermentation process of ASAZUKE cabbage (cabbage preserved with salt and malt). Also, Itabashi *et al.*[3,4,5,6] investigated the effect of various additions of lactic acid bacteria and organic acids on the quality of fermentation in SUNKI pickling.

In our previous study[9] on NISHINZUKE, we investigated the effect on product quality of the salt concentration and temperature of fermentation, with the aim of controlling quality during the fermentation process and in the distribution stage.

In the present study, we investigated the effect of the addition of lactic acid

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bacteria starters and organic acids on the activity of microorganisms in the fermentation process of NISHINZUKE under low salt concentration conditions.

Materials and Methods

Materials

NISHINZUKE was prepare by the following method commonly used in most homes, which was also described in our previous report[9]. Commercially available ingredients were used. Daikon (Japanese radish) and cabbage were sliced into 3 cm and 5 cm sections, respectively, and carrots and ginger were cut into fine strips. Filleted herring was soaked in tap water for 12 hours and after rinsing of the surface was cut into 4 cm thin slices. Commercial KOJI for pickling (Fukuyama Co.) was evenly ground and added. The following quantities were used. Daikon : 420 g ; cabbage : 440 g ; carrots : 30 g ; ginger : 15 ; koji : 45 g ; and filleted herring : 50 g. All the ingredients were first put into a large container and mixed well, and the mixture was then divided into 2 kg containers, and lactic acid bacteria starters and organic acids were added. The containers were then placed in cold storage (5°C) for fermentation, and samples were taken at various time intervals for analysis.

Preparation of the Lactic Acid Bacteria Starters

Leuconostoc mesenteroides (RGUF2610) and *Lactobacillus casei* (RGUR2651), which are preferred strains in the fermentation process of NISHINZUKE, were chosen as the lactic acid bactera starter strains. These two strains are proliferative at low temperatures. Cabbage (400 g), Daikon (400 g) and carrots (100 g) were boiled in 1000 mℓ of pure water and used for the preparation of the culture medium for the starter. The media cultured from the two bacterial strains were diluted in extracted solutions containing identical compositions, and added evenly so the initial bacteria count for the pickling ingredients would be $10^4 g^{-1}$.

Bacterial Counts

The bacteria count in each flora was measured basically by the pour-plate method. In other words, at the end of each fermentation process, a 50 g sample was collected in a sterilized stomacher bag, to which 450 mℓ of sterilized physiological saline solution was added and homogenized by stomacher.

Total bacteria (SPC)

For the measurement of the total bacteria count, the pour-plate method was carried out using a modified agar culture medium (meat extract 0.2%, peptone 0.2%, powder agar 1.5%, pH 7.2) to inhibit proliferation of lactic acid bacteria. This was cultured at 32°C for 48h and the bacteria count was calculated from the formed colonies.

Total lactic acid bacteria

Dry sterilized calcium carbonate (precipitative type) was added to a plate count medium with BCP (Eiken) to a level of 0.5% of the medium, and the plate was cultured for 72h at 32°C. Those bacteria that formed a transparent ring at the

rim of a colony were counted as acid-producing bacteria, and for other suspected colonies, the bacteria were confirmed by the catalase test and Gram staining.

Lactobacilli

Six mℓ of sterilized 4M acetic acid buffer solution (pH 5.3) was added to 100 mℓ of sterilized soluble acetate agar[14] base culture, and after culturing for 72-96h at 32°C, the number of formed colonies were counted. For other suspected colonies, the bacteria were confirmed by the catalase test and Gram staining. Moreover, in order to prevent the growth of aspergilli which was added to the total bacteria and lactic acid bacteria count media, we added Kabicidin (Nihon Seiyaku Co.) to the media at a rate of 100 mg to 1000 mℓ of medium.

Gram negative bacteria

A CVT agar medium was cultured at 28°C for 48h using the pour-plate method. The large red colonies were counted as Gram negative bacteria, and the suspected cases were confirmed by Gram staining.

Yeast

To a potato dextrose agar medium (Eiken), 10% tartaric acid was added, and the pH was adjusted to 3.8. Using the pour-plate method, the medium was cultured at 28°C for 72h, and confirmation of the yeast and mold was carried out by unaired visual inspection and microscopically.

Total Acid Volume and pH

One hundred of pure water was added to 100 g of the sample and was extracted over a 5h period while undergoing stirring in a refrigerator. The filtrate was neutralization titration to 0.1N NaOH using an automatic titration apparatus (Mitsubishi Kasei GT-05), and the total acid volume and pH were measured.

Measurement of Organic Acids

To 5 mℓ of a solution extracted in the same way as for the acidity measurement, 5 mℓ of acetonitrile was added, mixed and centrifuged (8000 rpm, 20 min). The supernatant was analyzed by HPLC (Waters organic acid column 7.8×300 mm). Also, malic acid and acetic acid were both analyzed using the Enzymatic Food Analysis F kit (Boehringer Mannheim Co.).

Identification of Lactic Acid Bactera

Isolated bacteria from the second week of NISHINZUKE fermentation were randomly isolated from the above-mentioned total lactic acid bacteria counting plate. The colonies were then streak-cultured in the plate medium, and identification tests were examined by the usual method for purely separated lactic acid bacteria.

Results and Discussion

Changes in Bacteria Flora in the Fermentation Process

The effect of the salt concentration on the acctivity of bacteria in the fermentation process is shown in Fig. 1. [A] shows 2.0% and [B] shows 3.5% salt

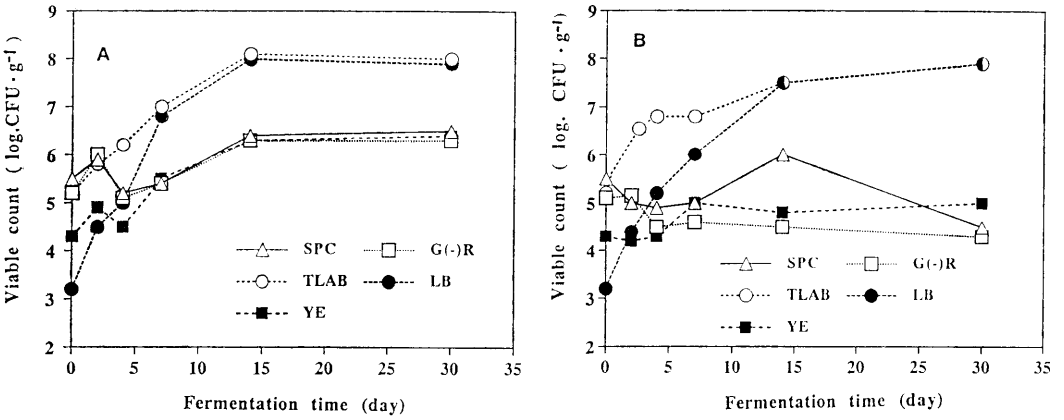

Fig. 1. Changes in microbial flora during the fermentation of NISHINZUKE at 5°C.

[A]: 2.0% NaCl. [B]: 3.5% NaCl.

Symbols: —△— SPC, total bacteria
--□-- G(−)R, Gram negative bacteria
--○-- TLAB, total lactic acid bacteria
—●— LB, lactobacilli
—■— YE, yeast

concentration. The bacteria count in the early stage of the pickling process was $10^5 g^{-1}$ for the total bacteria count, $10^5 g^{-1}$ for the Gram negative bacteria count and $10^4 g^{-1}$ for yeast. The total lactic acid bacteria count was $10^5 g^{-1}$ and the lactobacilli count was $10^3 g^{-1}$. Here, the total lactic acid bacteria includes both lactococci and lactobacilli, and the fact that the lactic acid bacteria count in the early stage of fermentation was less than $10^3 g^{-1}$ clearly indicates that the lactic acid bacteria derived from the ingredients are mainly lactococci. The lactobacilli are thought to be derived from Koji as well as from the ingredients. However, the lactobacilli showed a rapid increase in the first 1 to 2 days of the fermentation process, and after 7 days, increased to more than $10^6 g^{-1}$ for both 2.0% and 3.5% salt concentrations. The proliferation was especially marked for low salt concentration. In the fermentation with a 3.5% salt concentration, the Gram negative bacteria and yeast decreased. This is attributed to the synergistic effect of the increase in lactic acid bacteria and decrease due to the salt concentration. All the bacteria were stabilized in a stationary state after 2 weeks of fermentation.

Fig. 2 shows the effect on the bacteria acivity in the fermentation process of a mixed culture solution containing *Leuconostoc mensenteroides and Lactobacillus plantarum* added so that the initial bacteria count would be $10^4 g^{-1}$.

The increase in both the total lactic acid bacteria and lactobacilli was faster for both salt concentration levels compared to the case with no additions (Fig. 1). The result of this is that there was almost no increase in putrefactive bacteria, such as the total bacteria and Gram negative bacteria. The Gram negative bacteria with 2.0% salt had an especially low count due to the production of lactic acid from the initial increase in lactic acid bacteria and due to the synergistic suppres-

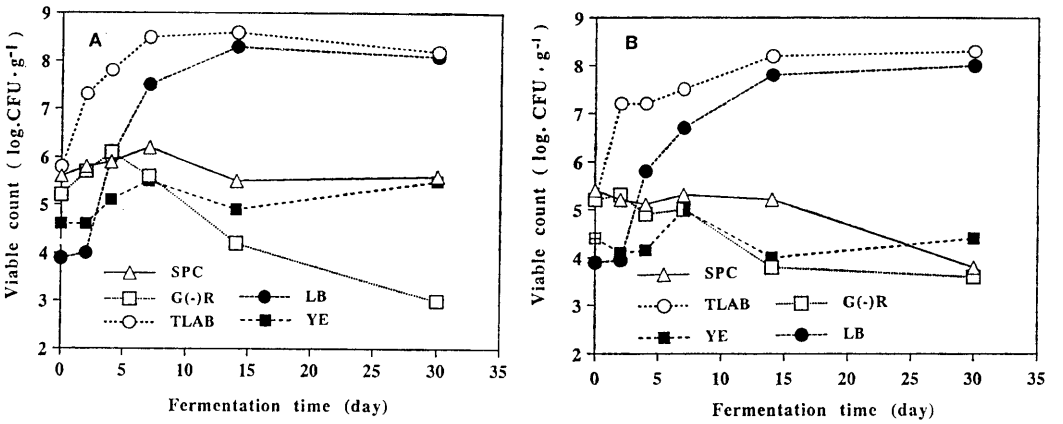

Fig. 2. Changes in microbial flora during the fermentation of NISHINZUKE supplemented with lactic acid bacteria starter at 5°C.

[A]: 2.0% NaCl. [B]: 3.5% NaCl.
Symbols as for Fig. 1.

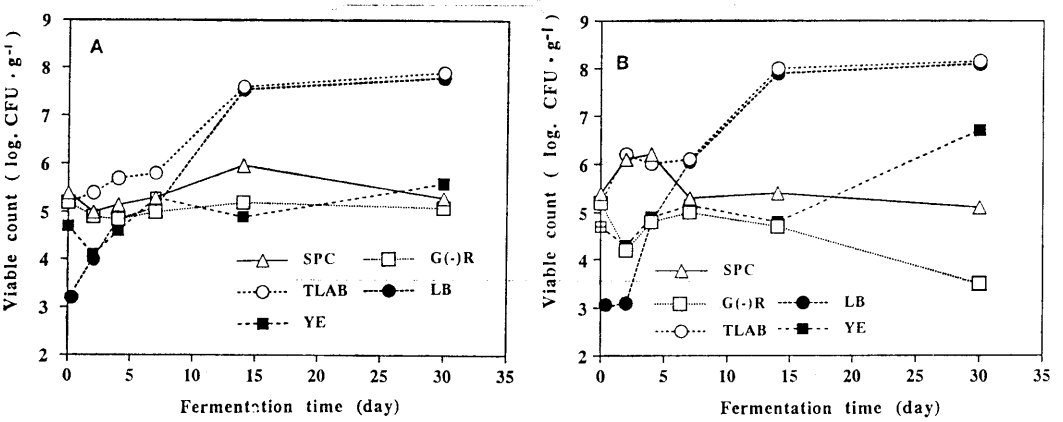

Fig. 3. Changes in microbial flora during the fermentation of NISHINZUKE supplemented with lactic acid (0.3%) at 5°C.

[A]: 2.0% NaCl. [B]: 3.5% NaCl.
Symbols as for Fig. 1.

sive effect of salt. This suppressive effect is also clear from following acidity results.

The production of lactic acid was accelerated, and putrefactive bacteria, such as Gram negative bacteria, were suppressed by the addition of lactic acid bacteria. Next, we investigated the effect of adding 0.3% lactic acid as an ingredient before the fermentation process on the activity of bacteria in fermentation. The results are shown in Fig. 3. The addition of lactic acid delayed the initial proliferation of total lactic acid bacteria and lactobacilli, and the bacteria count finally reached $10^5 g^{-1}$ one week after the start of fermentation for both 2.0% and 3.5% salt

concentrations. For the 3.5% salt concentration, the lactobacilli count actually showed a temporary decline in the early stage of fermentation. This suggests that lactobacilli which were used as starters had a relatively low resistance to salt. Despite the suppression of lactic acid bacteria proliferation. Gram negative bacteria and total bacteria proliferation were suppressed by the addition of lactic acid before fermentation, and never exceeded $10^6 g^{-1}$. As NISHINZUKE fermentation is carried out at a relatively low salt concentration, a rapid proliferation of lactic acid bacteria and control of putrefactive bacteria by salt concentration are considered to be very important for quality control.

Changes in the amounts of organic acid produced in the fermentation process are shown in Fig. 4. As the proliferation of lactic acid bacteria was suppressed by the addition of lactic acid before fermentation, only a small quantity of acid was produced. The acid production was highest with the addition of lactic acid and a 2.0% salt concentration, reaching 8 $m\ell \cdot 10\,g^{-1}$ after 2 weeks and then stabilizing. There was only a slow increase in the case of a 3.5% salt concentration.

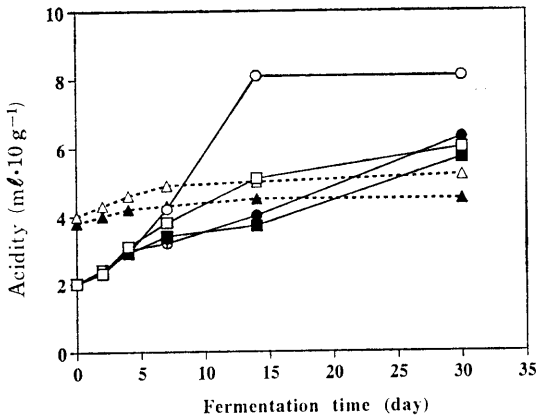

Fig. 4. Changes in the amounts of total acidity during the fermentation of "NISHINZUKE" at 5°C.

Symbols: 2.0% NaCl □, control (no additive)
○, lactic acid bacteria inoculated
△, lactic acid added (0.3%)
3.5% NaCl ■, control (no additive)
●, lactic acid bacteria inoculated
▲, lactic acid added (0.3%)

Changes in Organic Acids during the Fermentation Process

Various changes in organic acids have been observed during the fermentation process. The changes in malic acid, which were especially remarkable, are shown in Fig. 5. The initial concentration of malic acid was 85 $mg \cdot 100\,g^{-1}$ and decreased as the fermentation process proceeded. Malic acid decreased the most rapidly with the addition of lactic acid and 2.0% salt, almost disappearing after 7 days of fermentation.

The decrease in malic acid during the fermentation process has been pointed out in past studies[1,2,11]. It has been reported that lactic acid plays a role in the

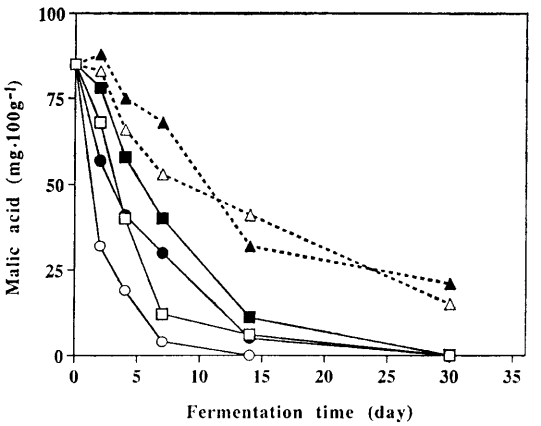

Fig. 5. Changes in the amounts of malic acid during the fermentation of "NISHINZUKE" at 5°C.

Symbols: 2.0% NaCl □, control (no additive)
○, lactic acid bacteria inoculated
△, lactic acid added (0.3%)
3.5% NaCl ■, control (no additive)
●, lactic acid bacteria inoculated
▲, lactic acid added (0.3%)

decrease of malic acid. Malic acid is thought to be decomposed into lactic acid and CO_2 through the action of lactic dehydrogenase of lactic acid bacteria. However, details of this mechanism are still not clear, and further investigation is needed.

Identification of Lactic Acid Bacteria

In order to clarify the dominant lactic acid bacteria in the fermentation process of NISHINZUKE, we randomly isolated lactic acid bacteria after 2 weeks of fermentation and carried out identification tests. The results are shown in Table 1.

The most predominant isolated bacteria were *Lactobacillus casei* (29.4%) and *L. plantarum* (10.6%), followed by hetero lactic acid bacteria, *L. cellobiosus* (12.0%) and *Leuconostoc mesenteroides* (33.0%), making up a total of 85%. A small quantity of *Lactobacillus brevis* and *L. coryniformis* was also isolated.

Kato *et al.*[7,8] investigated the lactic acid bacteria colonies during the storage period of salt-pickled Daikon, and found the main bacteria to be *L. curvatus*, *L. coryniformis* and *L. bavaricus*. Miyao *et al.*[12,13] reported that the main lactic acid bacteria in ASAZUKE cabbage (cabbage preserved with salt and malt) was *Leuconostoc meseneroidus*, which was similar to the our results for NISHINZUKE. They also reported that in SUNKI pickled with the addition of succinic acid, oxalacetic acid and malic acid, the dominant lactic acid bacteria varies according to the type of organic acid added[6]. Among the *Leuconostoc*, it has been verified that malic acid has a strong decompositional ability. Detailed investigation of this is now in progress. As was the case for the experiments in this study, it is thought that the fermentation process for NISHINZUKE differs slightly depending on the ingredients used. In our previous paper, we reported that the storage tem-

Table 1. Variable physiological and biochemical characteristics of lactic acid bacteria isolated from NISHINZUKE

Characteristics	Data for grouping on lactic acid bacteria isolated									
	A	B	C	D	E	F	G	H	I	J
Cell form	C	C	C	C	C	R	R	R	R	R
Catalase	−	−	−	−	−	−	−	−	−	−
Gas from glucose	+	+	−	−	−	+	−	−	−	+
Sugar fermentation										
Glucose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	·	·	+	+	+	+	+	+
Ribose	±	±	·	·	·	+	+	+	−	+
Sucrose	+	+	·	·	−	+	−	+	+	±
Arabinose	+	±	·	−	+	+	−	−	−	+
Trehalose	+	+	·	+	+	+	+	+	−	−
Lactose	−	±	+	−	+	−	+	+	±	−
Raffinose	−	−	−	−	+	±	±	+	−	−
Cellobiose	±	−	−	·	+	+	+	+	−	−
Mannitol	−	±	·	+	+	−	+	+	+	±
Maltose	+	+	·	·	+	+	−	+	+	+
Esculin	±	±	·	·	·	+	+	+	±	−
Mannose	+	+	·	·	·	−	+	+	+	−
Melezitose	·	·	·	·	−	−	+	−	−	±
Growth at										
10°C	+	+	+	+	+	−	+	+	+	+
15°C	+	+	+	+	+	+	+	+	+	+
40°C	−	−	+	+	+	+	+	+	±	+
45°C	−	−	−	−	+	+	±	±	−	−
50°C	−	−	−	−	−	−	−	−	−	−
Growth at pH										
4.2	−	−	+	−	+	·	+	+	−	+
4.8	−	+	+	−	+	+	+	+	±	+
8.5	·	·	+	+	+	·	·	·	·	+
9.6	·	·	−	−	+	·	·	·	·	+
Growth in NaCl										
6.5%	−	−	−	−	+	−	−	−	−	+
NH_3 from Arginine	·	·	+	−	+	+	−	−	+	+
Dextran from sucrose	+	−	−	−	−	+	−	−	−	+
Isomer of lactate	D	D	L	L	L	DL	L	DL	D	DL
Peptidoglycan type						non-DAP	non-DAP	DAP	non-DAP	non-DAP
No. of strains	68	9	3	6	1	25	61	22	8	1
(%)	32.9	4.3	1.1	2.9	0.5	12.0	29.1	10.6	3.8	1.9

A : *Leuconostoc mesenteroides* B : *Leuconostoc paramesenteroides*
C : *Lactococcus lactis* D : *Streptococcus iniae*
E : *Enterococcus facium* F : *Lactobacillus cellobiosus*
G : *Lactobacillus casei* H : *Lactobacillus plantarum*
I : *Lactobacillus coryniformis* J : *Lactobacillus brevis*

perature and salt concentration have a large effect on the fermentation process of NISHINZUKE[6]. The temperature in the fermentation process is especially important as it was found to be a main factor affecting the proliferation of lactic acid bacteria. As NISHINZUKE is made under low temperature conditions during the winter, the activity of lactic acid bacteria, which can grow in cold temperatures, has a great effect on the quality control in the fermentation process. Thus, it is important to be able to control the lactic acid bacteria. Also, as the salt concentration level suppresses not only harmful bacteria, but also the useful lactic acid bacteria, determining the appropriate quantity of lactic acid to add is very important for the fermentation process.

Summary

The effects of the inoculation of lactic acid bacteria starter and addition of organic acid to improve "NISHINZUKE" quality was investigated with 2.0% and 3.5% NaCl addition at 5°C.

The bacteria count in the early stage of the pickling process was $10^5 g^{-1}$ for the total bacteria count, $10^5 g^{-1}$ for the Gram negative bacteria count and $10^4 g^{-1}$ for yeast. The total lactic acid bacteria count was $10^5 g^{-1}$ and lactobacilli count was $10^3 g^{-1}$.

The lactobacilli showed a rapid increase in the first 1–2 days of the fermentation periods, and after 7 days increased to more than $10^5 g^{-1}$ for both 2% and 3.5% salt concentration.

Next we investigated the effect of adding *Leuconostoc mesenteroides* and *Lactobacillus plantarum* as an ingredient before the fermentation process on the activity of bacterial flora in fermentation. The increase in both the total lactic acid bacteria and lactobacilli was faster for both salt concentration levels compared to the case with no additions. The result was that there was almost no increase in putrefactive bacteria, such as the total bacteria and Gram negative bacteria.

In order to clarify the dominant lactic acid bacteria in the fermentation process of "NISHINZUKE", we randomly isolated lactic acid bacteria after 2 weeks of fermentation and carried out identification tests. The most predominant bacteria were *Lactobacillus casei* (29.4%) and *L. plantarum* (10.6%) followed by hetero lactic acid bacteria, *Leuconostoc mesenteroides* (33%) and *L. cellobiosus* (12.0%). A small quantity of *L. brevis* and *L. coryniformis* were also isolated.

Acknowledgment

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要　　約

ニシン漬の発酵品質におぼす乳酸菌スターターと乳酸の添加効果について，食塩濃度2.0%および3.5%で行なった。発酵初期におけるそれぞれの菌叢の菌数は，一般細菌数が$10^5 g^{-1}$，グラム陰性細菌数$10^5 g^{-1}$，酵母菌数$10^4 g^{-1}$であった。また，漬物発酵に重要な総乳酸菌数は$10^5 g^{-1}$であったが，乳酸桿菌数は$10^3 g^{-1}$と少なかった。しかし，発酵7日後には，食塩2.0, 3.5%濃度の両者において，$10^6 g^{-1}$以上に増殖した。また，ニシン漬の最優勢菌種である，*Leuconostoc mesenteroides, Lactobacillus casei*をスターターとして添加したものでは，総乳酸菌，乳酸桿菌ともに増殖が速く，乳酸生成が促進され，その結果一般細菌やグラム陰性細菌のような腐敗に関与する微生物を抑制した。

次に，ニシン漬の発酵過程における乳酸菌の関係を明らかにする目的で，発酵2週間後の乳酸菌を分離し，同定をした結果，ヘテロ乳酸菌の*Leuconostoc mesenteroides* (33%)，ホモ乳酸桿菌の*Lactobacillus casei* (29.4%)，*L. plantarum* (10.6%)，ヘテロ乳酸桿菌の*L. cellobiosus* (12.0%)などが優勢であり，これら4種で全体の85%を占めた。