Isolation and Identification of Lactic Acid Bacteria from Traditional Fermented Sauce in Southeast Asia

Iichiroh Ohhira, Chang Min Jeong, Taku Miyamoto and Kei Kataoka

Laboratory of Animal Products Technology, Faculty of Agriculture, Okayama University, Okayama, 700

Summary

A research was done to isolate and identify lactic acid bacteria from traditional fermented sauce foods such as belachan, budu, cinchuluk, pekasam, trassi, kicap, tauco, tempeyak and sambal belachan in Southeast Asia.

Lactic acid bacteria were dominant microbial flora in every sample. One hundred and thirty-five strains were physiologically characterized and classified into three genera and nine species of lactic acid bacteria.

Belachan was strains of Leuconostoc mesenteroides subsp. mesenteroides (6) and Lactobacillus plantarum (9). Budu contained L. Plantarum (14) and Streptococcus faecalis (1). Cinchuluk was found to contain Leuc. mesenteroides subsp. mesenteroides (6) together with Str. faecalis (9). Pekasam contained Str. faecium (9) and Leuc. mesenteroides subsp. mesenteroides (6). Trassi showed strains of Leuc. mesenteroides subsp. mesenteroides (4) and Str. faecium (11). Kicap contained Str. faecium (9) and Leuc. paramesenteroides (6) were isolated and identified. From tauco, L. coryniformis subsp. coryniformis (1) and Leuc. mesenteroides subsp. mesenteroides (14) were isolated and identified. Tempeyak was found to contain L. plantarum (10) together with Leuc. mesenteroides subsp. mesenteroides (3) and Str. faecalis (2). Constituent species of sambal belachan were L. casei subsp. casei (6), L. casei subsp. rhamnosus (1), L. plantarum (3), Str. faecalis (2), Str. faecium (2) and Str. gallinarum (1).

It was concluded that the distribution of lactic acid bacteria might be due to ingredients used for the traditional processing of fermented foods.

INTRODUCTION

The role of microflora in fermented foods is a very important issue on preservation, flavor, taste and nutrient (17). However, there are scarcely any reports discussing systematically on strains of extremely supreme.

We already conducted the identification of lactic acid bacteria isolated from native fermented foods being produced traditionally (17). In this research, we selected 9 kinds of traditional fermented sauce foods, representative in Southeast Asia such as belachan, budu, cinchuluk, pekasam, trassi, kicap, tauco, tempeyak and sambal belachan. Then we will report on the result of isolation and identification of lactic acid bacteria in aim to clarify the flora of lactic acid bacteria assumed to be developed widely on these samples.

METHODS

1. Test samples

We sampled 9 kinds in total such as: belachan made of small fishes and shrimps from the sea, salt and others categorized as sauce foods (17), budu, cinchuluk, pekasam with ingredients of freshwater
fishes and salt, trassi prepared with small fishes from the sea, kicap mainly with soybeans, cereals and salt, tauco, durian, tempeyak with a slight amount of salt, belachan and sambal belachan made of Chinese onions and red peppers. All samples were acquired from domestic business production factories, markets and private houses in Kelantan State, Pela State and Selangor State of West Malaysia. They were preserved refrigerated to prevent quality of the samples, brought back to the laboratory by air freight and kept until experiments at 5°C.

2. Measurement of the pH and salt content value

We measured the pH value with glass electrode pH meter and salt content value with salt concentration meter (NS-3P type, Sinar).

3. Bacterial counts and isolation of lactic acid bacteria

1) Bacterial counts of lactic acid bacteria

The above mentioned 9 kinds of test samples collected in West Malaysia was diluted from 1g or 1mL to 10⁹-fold and inoculated on BCP added plate count agar medium (Nissui) or MRS agar medium (Merck) prepared at pH5.4 as pour plate at 34°C ±1°C. We measured the bacterial counts of yellowing colonies after 3-day cultivation on BCP added plate count agar medium and, on the other hand, we measured the colony counts appeared after 2-day cultivation on MRS agar medium.

2) Isolation of lactic acid bacteria

After diluted the test sample 10-fold, we smeared with a platinum loop on BCP added plate count agar medium. Then, after 48-hour cultivation at 34°C ±1°C, we picked 15 representative colonies from each sample from yellowing of the medium and the form of the colony to isolate purely.

Also, we added 10ppm cycloheximide to inhibit the yeast growth on the medium we used for bacterial counts and isolation of lactic acid bacteria.

4. Identification test of isolated strains

Through investigation on the biochemical properties Table 3 to 5 show according to our previous reports (2,6) by sampling isolated strains, we tested the identification of lactic acid bacteria. For passage of lactic acid bacteria, we used a medium partially changed from Elliker medium (7).

The structure of the medium is: 2.0% trypton, 0.5% yeast extract, 1.0% glucose, 0.15% sodium chloride and 0.05% L-sodium ascorbic acid.

<table>
<thead>
<tr>
<th>Table 1 pH value, salt concentration and bacterial counts of traditional fermented sauce foods in Southeast Asia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Samples used</strong></td>
</tr>
<tr>
<td>Belachan</td>
</tr>
<tr>
<td>Budu</td>
</tr>
<tr>
<td>Cinchalu</td>
</tr>
<tr>
<td>Pekasam</td>
</tr>
<tr>
<td>Trassi</td>
</tr>
<tr>
<td>Kicap</td>
</tr>
<tr>
<td>Tauco</td>
</tr>
<tr>
<td>Tempeyak</td>
</tr>
<tr>
<td>Sambal belachan</td>
</tr>
</tbody>
</table>

* Salt concentration was estimated by using Sinar salt meter.

b KBP added plate count and MRS agar were used.
RESULTS

1. The pH value, salt content and lactic acid bacterial counts of test samples

Table 1 shows the pH value, salt content and lactic acid bacterial counts of test samples. The following is the pH value of each sample: belachan with pH 8.75, trassi with pH 7.25 and other samples in a range of pH 4 to pH 6.

On salt content volume, budu had the highest as 8.66%, sambal belachan and tempoyak had extremely low value as 0.32% and 0.12% respectively. It is assumed that the salt concentration decreased because sambal belachan is prepared with much volume of Chinese onion and red pepper.

On the other hand, the number of colonies on the medium measuring lactic acid bacterial count showed ranging from $2.0 \times 10^5$/g to $1.3 \times 10^{10}$/g on BCP added plate count agar medium and ranging from $3.0 \times 10^5$/g to $1.3 \times 10^8$/g on MRS agar medium. The bacterial counts of sambal belachan were the highest and those of tauco and trassi showed a tendency of low numbers. However, as each sample showed bacterial counts more than $10^5$, we assume that it is they are strains with lactic acid bacteria superior.

2. Property test results of isolated lactic acid bacteria

On a total number of 135 strains obtained by picking 15 strains from respective sample from the colonies appeared on the plate of BCP added plate count agar medium, we investigated Gram stain and observation of morphology of bacteria, whether they are aerobic or anaerobic, whether catalase grows or not, and change of limus milk (0.5% glucose content). As a result, all strains were anaerobic with Gram positive and negative in catalase test. Among them 44 strains were chain rods, aerobic and lactic acid bacteria because they are acid producing bacteria negative in catalase test.

3. Isolation test results of lactic acid bacteria isolated

We investigated on the identification compared to the new version of Bergey's after researching biochemical properties of above 135 strains isolated which were estimated as lactic acid bacteria. Table 3 to 5 show the results.

1) The genus Lactobacillus

Among the 135 isolated strains, 44 strains were of the genus Lactobacillus. The details are 6 strains of L. casei subsp. casei, 1 strain of L. casei subsp. rhamnosus, 1 strain of L. casei subsp. coryniformis and 36 strains of L. plantarum (Table 2). All strains grew at 15°C but did not grow at 45°C. Also, as a result of testing gas production from glucose using Gibson's medium ($\alpha$) to distinguish homo or hetero fermentation bacteria, most of the strains were negative. However, although in a part of strains slight cracks were shown, any clear gas production was admitted. The results of researching optical activity of producing lactic acid show L-type and DL-type.

Those 6 strains identified as L. casei subsp. casei among these strains showed the following contrast bacteria in saccharide fermentation test: 2 strains in aesculin, 5 strains of melibiose and 2 strains of sorbitol. However, they were identified as L. casei subsp. casei because other properties showed high similarity to contrast bacteria. The strain identified as L. casei subsp. rhamnosus was identical to contrast bacteria except for melibiose fermentation. One strain of L. casei subsp. coryniformis is different from contrast bacteria on melezitose or ribose fermentation. 36 strains identified L. plantarum are 6 strains of aesculin, 5 strains of melibiose, 7 strains of raffinose and 6 strains of sucrose different from contrast strains, they were identified as L. plantarum because other parts showed similarity.

2) The genus Streptococcus

Among 91 rods isolated, there are 46 strains in total distinguished as the genus Streptococcus producing morphology of bacteria and homo fermentation L(+) lactic acid. On saccharide fermentation 14 strains
isolated as *Str. faecalis*, those different from *Str. faecalis* are 2 strains of arabinose, 2 strains of cellobiose, 1 strain of glycerol and 2 strains of melibiose. For *Str. faecium*, among 31 strains, 5 strains of melezitose and 7 strains of salicin showed properties different from contrast bacteria in saccharide fermentation test. However, because in other part admitted high similarity, they were identified as *Str. faecium*.

One strain identified as *Str. gallinarum* showed growth on 0.1% methylene blue milk different from other 2 strains. Therefore, we identified it as *Str. gallinarum* which is the only strain showing negative in entero rods group described on Bergey's new version.

3) The genus *Leuconostoc*

Among the isolated bacteria, those strains assumedly belong to the genus *Leuconostoc* producing D (-) lactic acid in hetero fermentation are 45 strains. Among them, 39 strains were identified as *Leuc. mesenteroides* subsp. *mesenteroides* and 6 strains were identified as *Leuc. paramesenteroides*. Any strain does not grow at pH 4.8 but they coagulated

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Table 2  The characteristics of the strains of *Lactobacillus* species

<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>L. casei</em> subsp. <em>casei</em></th>
<th><em>L. casei</em> subsp. <em>rhamnosus</em></th>
<th><em>L. casei</em> subsp. <em>coryniformis</em></th>
<th><em>L. plantarum</em></th>
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<tbody>
<tr>
<td>No. of isolates</td>
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<td>1</td>
<td>36</td>
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<td>±</td>
<td>±</td>
<td>±/–</td>
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<td>NH₃ from arginine</td>
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<td>–</td>
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<td>Growth at 25°C</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid from</td>
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<td></td>
<td></td>
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<tr>
<td>Amygdalin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Esculin</td>
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<td>+</td>
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<td>+</td>
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<td>Mannitol</td>
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<td>Mannose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melezitose</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Melibiose</td>
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<td>+/–</td>
</tr>
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<td>–</td>
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<td>+</td>
<td>+/–</td>
</tr>
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<td>Trehalose</td>
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<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactic acid isomer</td>
<td>L (+)</td>
<td>L (+)</td>
<td>DL</td>
<td>DL</td>
</tr>
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</table>

+: positive, ±: weak positive, –: negative
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Str. faecalis</th>
<th>Str. faecium</th>
<th>Str. gallinarum</th>
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<tbody>
<tr>
<td>No. of isolates</td>
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<tr>
<td>Gas from glucose</td>
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<td>–</td>
</tr>
<tr>
<td>Growth in 6.5% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at pH 9.6</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hyppurate hydrolysis</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine hydrolysis</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in 0.1% methylene blue milk</td>
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<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid from</td>
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<td></td>
</tr>
<tr>
<td>Amygdalin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+/–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+/–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Galactose</td>
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<td>+</td>
<td>+</td>
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<td>Glucose</td>
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</tr>
<tr>
<td>Glycerol</td>
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<td>±</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
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<td>Mannitol</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+</td>
<td>+/–</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+/–</td>
<td>+</td>
<td>+</td>
</tr>
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<td>Ribose</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Salicin</td>
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<td>+/–</td>
<td>±</td>
</tr>
<tr>
<td>Sorbitol</td>
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</tr>
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<td>Sorbose</td>
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<tr>
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<tr>
<td>Lactic acid isomer</td>
<td>L (+)</td>
<td>L (+)</td>
<td>L (+)</td>
</tr>
</tbody>
</table>

+ : positive, ± : weak positive, – : negative

There are also strains that show different character from the contrast bacteria.

STUDY

We identified 135 strains of lactic acid bacteria isolated from 9 samples of fermented sauce foods widely utilized in daily food life which have been produced traditionally in the Southeast Asia. As a result, they were isolated to 9 species of 3 genera. When we divide those 9 strains identified in isolation sources, 9 strains of *L. plantarum* and 6 strains of *Leuc. mesenteroides* subsp. *mesenteroides* were isolated from belachan. Merican (10) clarifies the existence of the genus *Pediococcus* and the genus *Lactobacillus* in the lactic acid bacteria isolated from
### Table 4: The characteristics of the strains of *Leuconostoc* species

<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>Leuc. mesenteroides</em> subsp. <em>mesenteroides</em></th>
<th><em>Leuc. paramesenteroides</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolates</td>
<td>39</td>
<td>6</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in 6.5% NaCl</td>
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<td>+</td>
</tr>
<tr>
<td>Growth at pH 4.8</td>
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<td>−</td>
</tr>
<tr>
<td>Yeast glucose litmus milk</td>
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<td>+</td>
</tr>
<tr>
<td>Acid from</td>
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<td></td>
</tr>
<tr>
<td>Amygdalin</td>
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<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
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<td>+</td>
</tr>
<tr>
<td>Arbutin</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Fructose</td>
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<td>Galactose</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Lactose</td>
<td>±/−</td>
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<td>Salicin</td>
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</tr>
<tr>
<td>Sucrose</td>
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<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
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<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lactic acid isomer</td>
<td><strong>D (−)</strong></td>
<td><strong>D (−)</strong></td>
</tr>
</tbody>
</table>

+ : positive, ± : weak positive, − : negative

20 samples of belachan. Also, Campbell-Platt (7) tells that from bagoong of the Philippines similar to the raw materials produced and its preparation method of belachan isolated the genus *Pediococcus*. This is believed to act an important role in forming flavor of this food. However, any report describes on the strains of lactic acid bacteria.

The lactic acid bacteria isolated from budu were 2 species as 14 strains of *L. plantarum* and 1 strain of *Str. faecalis*. Although there is a research report on budu by Karim (11), the content only describes mainly on its biochemical properties and preparation method, but not on microflora. From cinchauluk, 2 species as 6 strains of *Leuc. mesenteroides* subsp. *mesenteroides* and 9 strains of *Str. faecalis* were isolated. From pekasam, 6 strains of *Leuc. mesenteroides* subsp. *mesenteroides* and 9 strains of *Str. faecium*, or from trassi 4 strains of *Leuc. mesenteroides* subsp. *mesenteroides* and 11 strains of *Str. faecium* were isolated.

Unlike the sauce foods prepared with above animal raw materials, kicap, tauco and tempoyak are prepared with vegetable raw materials. From kicap, 6 strains of *Leuc. paramesenteroides* and 9 strains of *Str. faecium* were isolated. It is said that the row material forming this product and its preparation method are quite similar to those of shoyu or soy sauce (12). The review by Yong *et al.* (13 - 16) remark that *Ped. halophilus* and *L. delbrueckii* are isolated from *moromi* or unrefined sake. Yokotsuka (17) also isolated and identified *Ped. halophilus* from *moromi*. Also, Lee *et al.* (18) reports the existence of *Ped.*
halophilus and Leuc.mesenteroides from meju, a product similar to kicap in Korea. From tauco, one strain of L.coryniformis subsp. coryniformis and 14 strains of Leuc.mesenteroides subsp. mesenteroides were isolated. Winarno et.al. (19) declares the existence of L.delbrueckii in tauco. Also, from tempyoyak isolated 10 strains of L.plantarum, 3 strains of Leuc.mesenteroides subsp. mesenteroides and 2 strains of Str.faecalis.

From sambal belachan produced together with animal and vegetable raw materials, 6 strains of L.casei subsp. casei, 1 strain of L.casei subsp. rharnnosus, 3 strains of L.plantarum, 2 strains of Str.faecalis, 2 strains of Str.faecium and 1 strain of Str.gallinarum were isolated.

There are scarcely any reports on microflora of fermented foods we tested this time, especially the flora of lactic acid bacteria. However, test samples were made through the steps of natural fermentation. It is assumed that the lactic acid bacterial flora distributed in the product are influenced by the raw materials or materials constructing these products. In the lactic acid bacteria isolated acutually, strains distributed widely in nature (20) such as L.plantarum, L.casei, Str.faecalis, Str.faecium and Leuc.mesenteroides were isolated. However, there is a tendency that a number of lactic acid bacteria are distributed in comparatively wide on the food the raw materials or materials ranging from animal to vegetable forming such products. The example is named sambal belachan.

Since above mentioned budu, cinchaluk and kicap possess high salt content volume (Table 2), we assume the existence of strains of the genus Pediococcus, which are halotolerant lactic acid bacteria. But this time, it was not isolated. We need to investigate further because we may think that the structure of medium for isolation would involve.

CONCLUSION

A research was done to isolate and identify lactic acid bacteria from nine samples of typically traditional fermented sauce foods in Southeast Asia, such as belachan, budu, cinchaluk, pekasam, trassi, kicap, tauco, tempyoyak and sambal belachan.

One hundred and thirty-five strains were physiologically characterized and classified into three genera and nine species of lactic acid bacteria. From belachan with animal raw materials isolated strains of Lactobacillus plantarum (9) and Leuconostoc mesenteroides subsp. mesenteroides (6). From budu L.plantarum (14) and Streptococcus faecalis (1). From cinchaluk isolated Leuc.mesenteroides subsp. mesenteroides (6) together with Str.faecalis (9). From pekasam isolated Leuc.mesenteroides subsp. mesenteroides (6) and Str.faecium (9). From trassi isolated strains of Leuc.mesenteroides subsp. mesenteroides (4) and Str.faecium (11).

From kicap using vegetable raw materials contained Leuc.paramesenteroides (6) and Str.faecium (9) were isolated and identified. From tauco, isolated L.coryniformis subsp. coryniformis (1) and Leuc.mesenteroides subsp. mesenteroides (14), from tempyoyak was found to contain L.plantarum (10) together with Leuc.mesenteroides subsp. mesenteroides (3) and Str.faecalis (2). From sambal belachan produced with both animal and vegetable raw materials, L.casei subsp. casei (6), L.casei subsp. rharnnosus (1), L.plantarum (3), Str.faecalis (2), Str.faecium (2) and Str.gallinarum (1) were isolated and identified. Traditional fermentation sauce foods stand in three kinds when divided roughly according to the raw material structure: animal, vegetable and mixed with both animal vegetable raw materials. It was concluded that the distribution of lactic acid bacteria might be due to ingredients used for the traditional processing of fermented foods. Those fermented foods made from animal and vegetable mixed possess a tendency of distribution of relatively many species.
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