

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

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Summary

A research was done to isolate and identify lactic acid bacteria from traditional fermented sauce foods such as *belachan*, *budu*, *cinchaluk*, *pekasam*, *trassi*, *kicap*, *tauco*, *tempoyak* 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). Cinchaluk 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. Tempoyak 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. gallinaum* (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 (1). 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 (2). In this research, we selected 9 kinds of traditional fermented sauce foods, representative in Southeast Asia such as belachan, budu, cinchaluk, pekasam, trassi, kicap,

tauco, tempoyak 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 (3-5), budu, cinchaluk, 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, tempoyak 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, Pera 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 (26) 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 1 pH value, salt concentration and bacterial counts of traditional fermented sauce foods in Southeast Asia

Samples used	pH	NaCl ^a (%)	Lactic acid bacterial counts	
			BCP ^b	MRS ^b
Belachan	8.75	1.77	6.1 × 10 ⁸ /g	3.5 × 10 ⁸ /g
Budu	5.02	8.66	2.0 × 10 ⁶ /ml	2.0 × 10 ⁷ /ml
Cinchaluk	5.72	4.65	7.0 × 10 ⁶ /g	2.0 × 10 ⁷ /g
Pekasam	5.67	1.51	1.9 × 10 ⁹ /ml	6.7 × 10 ⁸ /ml
Trassi	7.25	2.48	4.0 × 10 ⁶ /g	5.0 × 10 ⁶ /g
Kicap	5.93	5.82	3.0 × 10 ⁷ /ml	9.0 × 10 ⁷ /ml
Tauco	5.74	1.34	2.0 × 10 ⁶ /g	3.0 × 10 ⁶ /g
Tempoyak	4.15	0.12	9.0 × 10 ⁷ /g	1.6 × 10 ⁸ /g
Sambal belachan	4.25	0.32	1.3 × 10 ¹⁰ /g	6.9 × 10 ⁸ /g

^a 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 pH8.75, trassi with pH7.25 and other samples in a range of pH4 to pH6.

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 $2.0 \times 10^6/g$ 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 's, 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 litmus 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.coryniformis* 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 (8) 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.coryniformis* 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

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

Characteristics	<i>L. casei</i> subsp. <i>casei</i>	<i>L. casei</i> subsp. <i>rhamnosus</i>	<i>L. coryniformis</i> subsp. <i>coryniformis</i>	<i>L. plantarum</i>
No. of isolates	6	1	1	36
Gas from glucose	-	±	±	±/-
NH ₃ from arginine	-	-	-	-
Growth at 15°C	+	+	+	+
Growth at 25°C	-	-	-	-
Acid from				
Amygdalin	+	+	-	+
Arabinose	-	-	-	+
Cellobiose	+	+	-	+
Esculin	+/-	+	-	+/-
Fructose	+	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Gluconate	+	+	+	+
Lactose	+/-	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Mannose	+	+	+	+
Melezitose	+	+	+	+
Melibiose	+/-	+	+	+/-
Raffinose	-	-	+	+/-
Rhamnose	-	+	+	-
Ribose	+	+	+	+
Salicin	+	+	+	+
Sorbitol	+/-	+	+	
Sucrose	+	+	+	+/-
Trehalose	+	+	-	+
Xylose	-	-	-	-
Lactic acid isomer	L (+)	L (+)	DL	DL

+ : positive, ± : weak positive, - : negative

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 pH4.8 but they coagulated

Table 3 The characteristics of the strains of *Streptococcus* species

Characteristics	<i>Str. faecalis</i>	<i>Str. faecium</i>	<i>Str. gallinarum</i>
No. of isolaten	14	31	1
Gas from glucose	-	-	
Growth in 6.5% NaCl	+	+	+
Growth at pH 9.6	+	+	+
Hyppurate hydrolysis	+	+	+
Arginine hydrolysis	+	+	+
Growth in 0.1% methylene blue milk	+	+	-
Acid from			
Amygdalin	+	+	+
Arabinose	+/-	+	+
Cellobiose	+/-	+	+
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Glycerol	+/-	±	+
Lactose	+	+	+
Maltose	+	+	±
Mannitol	+	+	+
Mannose	+	+	±
Melezitose	+	+/-	+
Melibiose	+/-	+	+
Ribose	+	+	+
Salicin	+	+/-	±
Sorbitol	+	-	+
Sorbosose	-	-	-
Sucrose	+	+	+
Lactic acid isomer	L (+)	L (+)	L (+)

+ : positive, ± : weak positive, - : negative

glucose content litmus milk yeast extract was added.

And they were categorized as 39 strains which does not show growth and 6 strains which show growth on 6.5% salt content medium. Among these, those 39 strains which did not show any growth on 6.5% salt content medium showed the identical character to the contrast bacteria *Leuc. mesenteroides* subsp. *mesenteroides* but have weak fermentation of galactose. On the other hand, other 6 strains showing growth on 6.5% salt content medium were identified as *Leuc. paramesenteroides*, which is said to have high halo-tolerance in the genus *Leuconostoc* (9). However, on saccharide fermentation, they are 2 strains of mannose, 2 strains of melibiose and 6 strains of salicin.

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

Characteristics	<i>Leuc. mesenteroids</i> subsp. <i>mesenteroides</i>	<i>Leuc. paramesenteroides</i>
No. of isolates	39	6
Gas from glucose	+	+
Growth in 6.5% NaCl	-	+
Growth at pH 4.8	-	-
Yeast glucose litmus milk	+	+
Acid from		
Amygdalin	+	+
Arabinose	+	+
Arubutin	-	-
Cellobiose	±	±
Fructose	+	+
Galactose	±	±
Glucose	+	+
Lactose	±/-	-
Maltose	+	+
Mannitol	+	+
Mannose	+	+/-
Melibiose	+/-	+/-
Reffinose	+	+
Salicin	+	+
Sucrose	+	+
Trehalose	+	+
Xylose	-	-
Lactic acid isomer	D (-)	D (-)

+ : positive, ± : weak positive, - : negative

20 samples of belachan. Also, Campbell-Platt (1) 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 cinchaluk, 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, taucu 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 raw 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 tempoyak 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. *rhamnosus*, 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 actually, 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, tempoyak 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 tempoyak 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. *rhamnosus* (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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