

## 2. Original Reports

# Identification of Lactic Acid Bacteria Isolated from Traditional Side-dish Fermented Foods in Southeast Asia

Ichiroh Ohhira, Chon Chan Min, Taku Miyamoto  
Kei Kataoka and Toshitaka Nakae

*Division of Applied Production Technology,  
Graduate School of Natural Science and Technology,  
Okayama University, Okayama, 700 Japan*

### Summary

Forty-one isolates were physiologically characterized and classified into seven species of lactic acid bacteria.

*Dadiah* was found to contain seven strains of *Streptococcus lactis* together with two strains of *Leuconostoc lactis*. *Dosai* contained two strains each of *Lactobacillus coryniformis* subsp. *coryniformis* and *Streptococcus bovis*. Among isolates from *idli* one strain was *Lactobacillus casei* subsp. *pseudoplanarum*, whereas two strains which showed only pairs of cells in morphology were found to be *Pediococcus halophilus*. *Tape* showed the presence of *Lactobacillus casei* subsp. *pseudoplanarum* which was slightly different in physiological properties. Only *Streptococcus faecalis* was present in *tempeh*. However, two strains from *dosai* and five strains from *idli* were not identified at the species level.

### INTRODUCTION

The role of microbial flora on fermented foods is extremely important issues such as preservation, aroma, taste, and nutrient (1,2,3). We are under testing isolation and identification of lactic acid bacteria from local fermented foods produced traditionally in especially in Southeast Asia in aim of isolating lactic acid bacteria from nature which has specific characteristic in cultivation and metabolism and we are conducting applied research of beneficial bacteria (4,5,6)

In this research, we tested the identification of strains, lactic acid bacteria isolated from traditional side-dish fermented foods in Southeast Asia (*dadiah*, *dosai*, *tape* and *tempeh*) in our previous research.

### METHODS

We tested the identification of lactic acid bacteria by studying physiological attribute test by sampling 41 strains of lactic acid bacteria isolated from traditional side-dish fermented foods in Southeast Asia. The agar for subculture and test of lactic acid bacteria is modified Elliker culture broth which part is changed from Elliker culture broth (7). The structure of the culture broth is: distilled water is added making 1000mL in total to 20g tripton, 5g yeast extract, 10g glucose, 4g sodium chloride, 1.5g acetic acid sodium and 0.5g L-ascorbic acid sodium. After preparing to pH 6.8-7.0, high-pressure sterilized for 15 minutes at 121°C.

#### 1) Optical activity of produced lactic acid

Under the existence of lactic acid dehydrogenase (LDH), we judged optical activity of produced lactic acid ( $\beta$ ) by measuring absorbance of NADH formed by acidifying lactic acid by nicotinamide adenine dinucleotide (NAD) with 340mm wave length. We used F-kit of Boehringer Mannheim Yamanouchi Pharmaceutical Co., Ltd.

#### 2) Approval of growing temperature

We inoculated each strain to modified Elliker culture broth and fermented for 5 days at other than 35°C, 10°C, 15°C, 37°C, 40°C and 45°C. Then we observed their growth.

#### 3) Formation of ammonium from arginine

We inoculated in modified Elliker culture broth which 0.3-0.5% L-arginine hydrochloric acid hydrochloride was added. After 7-day cultivation, we added 1mL NESSLER reagent to 4mL of those. We considered those precipitated fresh yellow or red brown positive.

#### 4) Identification of homo and hetero lactic acid fermentation ( $\beta$ )

11g glucose is dissolved into 160mL litmus milk and heated (a). Then 0.6g powdered gelatin, 0.4g peptone, 0.4g meat extract, 0.2g sodium chloride are dissolved making to be 40mL (b). And, 0.56g yeast extract is dissolved to 20mL tomato juice (filtrated in a filter) and heated (c). (a),(b) and (c) are mixed to be prepared to pH 7.0. They were divided in 10mL in each test tube and high-pressure sterilized.

Above culture broth is dissolved and kept at 43°C-45°C. 1mL of culture solution of previously subcultured modified Elliker culture broth is inoculated and mixed well, then solidified. Then poured 1.5% liquid gelatin to the top making its height about 1.5cm, solidified and cultivated at 35°C.

In case of hetero lactic acid bacteria, they produce vigorous gas and push up gelatin tap but in case of homo lactic acid bacteria, such change is not observed.

#### 5) Growth at 6.5% sodium chloride content culture broth

Sodium chloride is added to modified Elliker culture broth to be 6.5% constituency, filtrated and sterilized. Then strains are inoculated and cultivated for 7 days at 35°C. Thus we judged if they grew or not.

#### 6) Growth at pH 9.6

Prepared to be pH 9.6 modified Elliker culture broth was filtrated and sterilized. Then strains are inoculated and cultivated for 5 days at 35°C. Thus we judged if they grew or not.

#### 7) Resolution power of hippuric acid soda

After sterilizing 10mL modified Elliker culture broth where 0.1% hippuric acid soda is added, we inoculate strains and cultivate it for 7 days at 35°C. 1mL of 50% H<sub>2</sub>SO<sub>4</sub> solution is added to 1mL supernatant fluid. We considered positive those being produced benzoic acid needle-like crystals by hydrolysis of hippuric acid soda.

#### 8) Change in litmus milk

0.5% glucose and 0.5% yeast extract were added and dissolved in litmus milk. Then high-pressure sterilized for 20 minutes at 110°C. Each strain is inoculated and cultivated for 7 days at 35°C. By judging change of litmus and situation of coagulation, considered positive those changed into red, reduced and coagulated.

#### 9) Fermentation of saccharide

We made a basic culture broth by adding 0.006% Promcresol Purple to modified Elliker culture broth not including glucose and preparing to be pH 6.8. 0.5% cellobiose, galactose, glycerol, gluconate potassium, inulin, lactose, mannitol, melezitose, melibiose, raffinose, salicin, sorbisucrose and trehalose are added one by one species and dissolved. They were divided in 5mL to small test tubes. After sterilized for 20 minutes at 115°C, inoculated each strain and cultivated for 10 days at 35°C.

Also, for arabinose, fructose, mannose, ribose and sorbose, 5% solution is filtrated and sterilized, then added to be 0.5% constituency in above culture broth. After inoculated each strain 5mL as other saccharide as the culture broth, cultivated for 10 days at 35°C and observed their change in color.

RESULTS

In our previous research, we estimated strains producing negative acid in catalase test showing facultative anaerobe as lactic acid bacteria. They are Gram-positive rods or cocci in strains isolated from dadih, dosai, idli, tape and tempeh as traditional side-dish fermented foods from Southeast Asia. Then we selected 41 strains applying to the appropriate condition. We researched on physiological character of these strains and tested identification of each strain comparing to the results shown on the new version of Bergey's (10).

Table 1 Differential characteristics of the genus *Lactobacillus* from traditional side-dish fermented foods in Southeast Asia

(I)

Species	Source	Strain No.	Morphology	Lactic acid isomer	Growth at		
					10°C	15°C	45°C
<i>L. coryniformis</i> subsp.	DOSAI  IDLI TAPE	7.13.  13. 1.2.3. 5.9.10 12.13. 14.15.	Rods in chains	DL	±	+	-
<i>coryniformis</i>							
<i>L. casei</i> subsp.							
<i>Pseudoplanatarum</i>							
<i>L. coryniformis</i> subsp.			Rods in chains	D-(-) L-(+)	/	+	/
<i>coryniformis</i> *							
<i>L. casei</i> subsp.			Rods in chains	DL	/	+	/
<i>Pseudoplanatarum</i> *							

(II)

NH <sub>3</sub> from arginine	Gas from glucose	Acid produced from																			
		Arabinose	Cellobiose	Fructose	Galactose	Gluconate	Glucose	Glycerol	Inulin	Lactose	Mannitol	Mannose	Melibiose	Melezitose	Raffinose	Ribose	Salicin	Sorbitol	Sorbose	Trehalose	Sucrose
-	±	/	+	+	+	±	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+
-	±	/	/	/	/	-	+	-	-	+	+	/	-	-	-	±	±	+	-	-	±
-	±	/	/	/	/	±	+	-	-	+	+	/	-	+	-	±	±	+	-	-	±
-	±	/	/	/	/	±	+	-	-	+	+	/	-	+	-	±	±	+	-	-	±
-	±	/	/	/	/	±	+	-	-	+	+	/	-	+	-	±	±	+	-	-	±
-	/	-	-	+	+	+	+	/	/	d	+	+	d	-	d	-	d	d	/	-	+
-	/	-	+	+	+	+	+	/	/	+	+	+	-	+	-	+	+	+	/	+	+

(+) : Positive.  
 (-) : Negative.  
 (±) : Slightly positive.  
 (/) : Not determined.  
 (d) : Variable.  
 (\*) : Data were adapted from Table 14.3 (p 1220), Table 14.7 (p 1227) and Table 14.8 (p 1228) of "BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY, Vol. 2".

1) The genus *Lactobacillus*

Among 41 strains of isolate, 13 strains were of the genus *Lactobacillus*. They are 2 strains from dosai, 1 strain from idli and 10 strains from tape. Table 1 shows identification test results in the bottom by quoting related items from "Bergey's Manual of Systematic Bacteriology Vol.2" in aim to compare each strain's test results and their score with standard strains by studying the physiological and biochemical character of these strains.

In the optical activity test of produced lactic acid, all strains shown in the Table 1 presented DL. And, to identify homo and hetero fermentation, we studied formation of gas from glucose by using Gibson's culture broth (9) and observed a slight crack in a part of each strain's culture broth.

Among these strains, 2 strains isolated form Dosai presented positive in serbiose and ribose fermentation on saccharide fermentation test. However, we identified them as *Lactobacillus coryniformis* subsp. *coryniformis* since they were identical to standard strains on other characters.

Table 2 Differential characteristics of the genus *Streptococcus* from traditional side-dish fermented foods in Southeast Asia

(I)

Species	Source	Strain No.	Motphology	Lactic acid isomer	Growth at		
					10°C	15°C	45°C
<i>Str. lactis</i>	DADIH	1.3.4. 7.8.9. 11.	Cocci, pairs and chains	L-(+)	+	+	-
<i>Str. bovis</i>	DOSAI	10.15.	Cocci and chains	L-(+)	±	±	-
<i>Str. faecalis</i>	TEMPEH	1.3.9. 10.12. 13.14. 15.	Cocci, pairs and chains	L-(+)	+	+	+
<i>Str. lactis</i> *			Cocci, chains	L-(+)	+	+	-
<i>Str. bovis</i> *			Cocci, chains	L-(+)	-	-	d
<i>Str. faecalis</i> *			Cocci, chains	L-(+)	+	+	+

(II)

Gas from glucose	Growth at 6.5% NaCl	Growth at 9.6 pH	Arginine hydrolysis	Hippurate hydrolysis	Acid produced from																			
					Arabinose	Cellobioss	Fructose	Galactose	Gluconate	Glucose	Glycerol	Inulin	Lactose	Mannitol	Mannose	Melibiose	Melezitose	Raffinose	Ribose	Salicin	Sorbitol	Sorbose	Trehalose	Sucrose
-	-	-	±	-	/	+	/	/	-	+	-	-	+	±	/	/	/	-	+	+	-	-	+	±
-	-	+	-	-	/	+	/	/	-	+	-	-	+	±	/	/	/	±	+	+	-	-	+	±
-	+	+	+	+	/	+	/	/	-	+	-	-	+	+	/	/	/	+	+	+	+	-	+	±
-	-	-	d	d	+	/	/	+	/	+	-	-	+	+	/	/	/	-	+	+	-	-	+	±
-	-	d	-	-	d	/	+	+	/	+	-	d	+	d	+	/	/	/	+	d	/	d	+	±
-	+	+	+	+	-	+	+	+	/	+	+	/	+	+	+	-	+	-	+	+	+	-	/	±

- (+): Positive.
- (-): Negative.
- (±): Slightly positive.
- (/): Not determined.
- (d): Variable.

(\*): Data were adapted from Table 12. 15 (p 1048), (p 1066), (p 1069) and (p 1064) of "BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY, Vol. 2".

On the other hand, 1 strain isolated from idli and 10 strains isolated from tape were showing extremely identical nature to the attribute we tested here. However, on growth temperature and saccharide fermentation, we had 5 groups when we classified those with identical nature. Especially, strain No.13 from idli was different from standard strains on gluconate and melezitose fermentation. Though other attribute showed identical nature to *L. casei* subsp. *pseudopiantarum*.

2) The genus *Streptococcus*

Among 41 strains of isolates, 28 strains except for rods showed applying to species of the genus *Streptococcus*. Table 2 shows identification test results of these strains and attributes of identically high standard strains.

In optical activity test of produced lactic acid, all strains showed L. However, on the growth temperature, growth under 6.5% sodium chloride, and saccharide fermentation, each strain showed difference and they were categorized in 3 species. Among those, 7 strains from dadih were observed to grow at 10°C, but not at 45°C. Saccharide fermentation was identical to standard strain of *Streptococcus lactis*.

Two strains from dosai were identified as bacteria identical to *Str. bovis* from their growth temperature and other attributes.

And, strains isolated from tempeh were able to grow both at 15°C and 45°C, with proteolytic activity of arginine and hipurric acid soda. They were observed to grow at 6.5% sodium chloride content culture broth and at pH 9.6. Saccharide fermentation was different from standard strains at glycerol fermentation but identified as *Str. faecalis* by other attributes.

3) The genus *Leuconostoc*

Among the isolates, the strain considered to correspond to the genus *Leuconostoc* were 2 strains from dadih. Table 3 shows a series of attributes in identification test results and nature of standard strains considered to be similar.

Table 3 Differential characteristics of the genus *Leuconostoc* from traditional side-dish fermented foods in Southeast Asia

(I)

Species	Source	Strain No.	Morphology	Lactic acid isomer	Growth at		
					10°C	15°C	37°C
<i>Leuc. lactis</i>	DADIH	14,15.	Chains	D-(-)	+	+	+
<i>Leuc. lactis</i> *			Chains and pairs	D-(-)	+	+	+

(II)

Gas from glucose	Yeast glucose litmus milk	Growth at 6.5% NaCl	Acid produced from																			
			Arabinose	Cellobiose	Fructose	Galactose	Gluconate	Glucose	Glycerol	Inulin	Lactose	Mannitol	Mannose	Melibiose	Melezitose	Raffinose	Ribose	Salicin	Sorbitol	Sorbose	Trehalose	Sucrose
+	+	-	±	-	+	/	-	+	-	-	+	-	/	+	±	-	±	-	-	-	+	±
+	+	-	-	-	+	+	/	+	/	/	+	-	d	d	d	d	/	d	/	/	+	+

- (+): Positive.
- (-): Negative.
- (±): Slightly positive.
- (/): Not determined.
- (d): Variable.

(\*): Data were adapted from Table 12.26 (p 1071) and Table 12.27 (p 1072) of "BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY, Vol. 2"

Table 4 Differential characteristics of the genus *Pediococcus* from traditional side-dish fermented foods in Southeast Asia

(I)

Species	Source	Strains No.	Morphology	Lactic acid isomer	Growth at		
					35°C	40°C	45°C
<i>P. halophilus</i>	IDL1	3.7.	Pairs	L-(+) D(-)	+	-	-
<i>P. halophilus</i> *			Tetrads, pairs.	L-(+) D(-)	+	-	-

(II)

Hippurate Hydrolysis	Gas from glucose	Growth at 6.5% NaCl	Arginine hydrolysis	Acid produced from																			
				Arabinose	Cellulose	Fructose	Galactose	Gluconate	Glucose	Glycerol	Inulin	Lactose	Mannitol	Mannose	Melibiose	Melezitose	Raffinose	Ribose	Salicin	Sorbitol	Sorbose	Trehalose	Sucrose
-	-	+	-	+	/	/	/	+	+	+	-	±	-	/	+	-	±	+	-	-	+	+	+
-	-	+	-	+	/	/	/	/	+	+	/	-	/	/	/	-	/	+	/	-	/	+	+

(+): Positive.

(-): Negative.

(±): Slightly positive.

(/): Not determined.

(d): Variable.

(\*): Data were adapted from Table 12.32 (p 1076) and Table 12.34 (p 1077) of "BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY, Vol. 2".

In optical activity test of produced lactic acid, they showed D. In growth temperature test, they grew fully in every range at 10°C, 15°C and 37°C. These 2 strains showed hetero lactic acid fermentation and coagulation of glucose contained litmus milk, yeast extract added. However, the growth under 6.5% sodium chloride was negative.

In saccharide fermentation test, fructose, glucose, lactose and trehalose were positive; arabinose, melibiose, melezitose, ribose and sucrose were quasi-positive. However, we estimated them as *Leuconostoc lactis* as other saccharide showed negative as standard strains.

4) The genus *Pediococcus*

Among the strains isolated, estimated strains as the genus *Pediococcus* were 2 strains from idli. Since their morphology was in pairs, we estimated them as species of the genus *Streptococcus* or *Leuconostoc* in our previous report (6). However, lactic acid produced L(+)- and D(-)-. In the growth temperature test, they showed growth at 35°C but not at 40°C and 45°C.

All strains had homo lactic acid fermentation and showed no production of ammonium from arginine. However, we identified them as *Pediococcus halophilus* from the growth at 6.5% sodium chloride existence and saccharide fermentation.

STUDY

We tested 41 strains of lactic acid bacteria isolated from 5 samples of side-dish fermented foods being fond of drinking and eating traditionally in Southeast Asia, studied their physiological and biochemical attribute and tested on identification. We classified them in total of 7 kinds of strains in reference to strains similar in their attributes presented in Bergey's new version (10). However, we could not identify 2 strains from dosai and 5 strains from idli in the attribute test in this research, so that we would need further investigation.

When we see identified strains from their isolate source, *Str. lactis* and *Leuc. lactis* were isolated from dadih. Since dadih is a fermented food prepared mainly milk, isolation of lactic acid bacteria for milk is estimated.

Mattick, A.T.R. *et.al* (11) isolated *Str. lactis* from dadih, Oxford, A.E. (12) isolated from *Str. cremoiris*, and Kodama, R. (13) isolated *L. plantarum*. Hamada, K. (14) isolated *Str. faecalis*, Pulusani, S.R. (19) isolated *L. acidophilus* and Pulusani, S.R. (19) isolated *Str. thermophilus*.

From dosai, *L. coryniformis* subsp. *coryniformis* and *Str. bovis* were isolated. The former is especially the lactic acid bacteria distributed widely in Plants Kingdom. Since dosai is a fermented food prepared from chickenpeas or *lens* (plants of the legume family) and rice powder, we estimated that it is from these raw materials.

Lewis, Y.S. *et.al.* (20) reports that they reported *L. delbrueckii*, *Leuc. lactis* and *Str. lactis* from the knead powder of dosai.

From idli, *L. casei* subsp. *pseudopplantarum* and *Pediococcus halophilus* were isolated.

Idli is a fermented food prepared from fenugreek (plants of the legume family) and similar in preparatory method of dosai. Mukherjee, S.K. *et.al* (21) reports that they isolated *Leuc. mesenteroides*, *Str. faecalis*, and *P. cerevisiae*.

From tape, isolated *L. casei* subsp. *pseudopplantarum* and *Pediococcus halophilus*. Tape is prepared from local rice (*Oryza saiva indica*) and a mixture of cassava. Tape is eaten after 3-day fermentation inside at static condition. Since this tape belongs to the group of knead powder, it is fully estimated the isolation of *L. casei* subsp. *pseudopplantarum* distributed widely in plants raw materials.

Though the research study of tape's lactic acid bacterial flora is very few, Okafor, N. (22) reports in his research that the existence of the genus *Lactobacillus* and the genus *Leuconostoc* take a great role in the process of static fermentation of tape.

From temph, isolated *Str. faecalis*. Tempeh is prepared from local soybeans which were rinsed in water for 2-3 days, steamed, spread in a wooden case, added ragi and fermented. Although there are many research studies of microflora of tempeh, many of them are on *Rhizopus* spp. but no literature on lactic acid bacterial flora is found. However, from soybeans fermented foods, there are many reports on isolation of *Str. faecalis* meaning that its existence is fully considered.

Among the raw material we tested here, *Str. lactis* and *Leuc. lactis* relating deeply to milk products are considered to be isolated since dadih is a fermented food prepared mainly from milk. On the other side, from vegetable fermented foods dosai, idli, tape and tempeh, isolated many *L. coryniformis*, mesophilic homo lactic acid rods *L. casei* and *Str. faecalis* which are said to distribute widely in Plantae.

And *P. halophilus* which is said to distribute widely in salted vegetable fermented foods for its characteristics, was isolated from 2 strains of idli.

## SUMMARY

A research was done to select 5 samples from representative traditional side-dish fermented foods of Southeast Asia as dadih, dosai, idli, tape and tempeh, isolated lactic acid bacteria from them and identified.

After examining physiologic and biochemical attribute of 41 strains isolated from lactic acid bacteria and classified them into 7 strains according to their similarity. As a result, 7 strains of *Streptococcus lactis* and 2 strains of *Leuconostoc lactis* were isolated from dadih.

From dosai, 2 strains from each *Lactobacillus coryniformis* subsp. *coryniformis* and *Streptococcus bovis* were isolated. Strains isolated from idli were 1 strain of *Lactobacillus casei* subsp. *pseudopplantarum* and 2 strains of *Pediococcus halophilus*.

Especially, the strain identified as *Pediococcus halophilus* was morphologically in pairs and other attributes showed the characteristic of the genus *Pediococcus*.

From tape, isolated 10 strains of *Lactobacillus casei* subsp. *pseudoplantarum* with slight changes in physiologic and biochemical attribute. On the other hand, isolates from tempeh were all *Streptococcus faecalis*.

However, we could not identify the species of 2 strains from dosai and 5 strains from idli.

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