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2. Original Reports

The Isolation and Identification of Lactic Acid Bacteria from Naturally Fermented Wild Plants and Fruits

Iichiroh Ohhira and Toshitaka Nakae

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

Summary

Studies were done to isolate and identify lactic acid bacteria from a naturally fermented mixture of four species of wild plants and seven species of wild fruits. Those plants and fruits are mugwort (*Artemisia montana*), sea tangle (*Laminaria japonica*), brown algae (*Hijikia fushiforme*), Chinese bayberry (*Myrica rubra*), wild vine (*Vitis coignetiae*), akebi (*Akebia quinata*), mulberry (*Morus australis*), wild strawberry (*Rubus buergeri*), oleaster (*Elaeagnus montana*), Chinese matrimony (*Lycium chinese*) and broad leafed plantain (*Plantago asiatica*).

The fermented mixture was prepared by fermentation in bottle with natural black sugar under natural weather for more than two years and extracted as a fermented juice.

The fermented juice was examined for counting and isolation of lactic acid bacteria in BCP plate count agar and modified ELLIKER's culture broth. It was found that the extract of fermented juice contained about 5.2×10^7/mℓ of lactic acid bacteria, which were identified as *Streptococcus faecalis* and *Streptococcus faecium*.

Food industries are showing a remarkable development these years. The volume of demand and supply is on a rising increase every year producing a variety of products where a part of them tends to go after a fashion. The form of food culture also changes gradually that people rely more and more on instant foods. However, these instant foods can never valued sufficient in regard to nutritious balance.

Then, considering natural food including vitamin, mineral and beneficial microbes we tend to lack in such instant foods, we conducted a development of fermented food using wild plants and fruits. As the ingredients for fermentation, we selected traditionally edible wild terrestrial or aquatic plant and wild fruits. Namely, Chinese bayberry (*Myrica rubra*), wild vine (*Vitis coignetiae*), akebi (*Akebia quinata*), wild strawberry (*Rubus buergeri*), mulberry (*Morus austrialis*), oleaster (*Elaeagnus montana*), Chinese matrimony (*Lycium chinese*), broad leafed plantain (*Plantago asiatica*), mugwort (*Artemisia montana*), algae (*Hijikia fusiforme*), sea tangle (*Laminaria japonica*) and others as fermentation mixed ingredients. These are extracted with natural muscovado sugar (originated in Okinawa, before refined), fermented and aged for more than two years under natural circulation. This study aimed these fermented products and screened microflora, especially lactic acid bacteria.

For the study, we referred documents from Nakae (*1,2*), Yano (*3*), Morichi (*4*), Inagami (*5*), Hosono *et.al.* (*6*), Mitsuoka (*7*) and others. We made it in consideration to isolation/identification of new lactic acid bacteria from nature especially and its usage; and more aggressive development of new products focusing on treatment effects on nutritious hygiene of lactic acid bacteria.

METHODS

1. Preparation of fermented extracts

We had 80kg of fermented products ingredients in total: 5kg Chinese bayberry, 5kg wild vine, 5kg akebi, 5kg wild strawberry, 10kg mulberry, 5kg oleaster, 5kg Chinese matrimony, 10kg broad leafed plantain, 10kg mugwort, 10kg algae and 10kg sea tangle. Chinese matrimony was used in powder form. And dried sea tangle and algae were wet and used on 3rd day after weighed. Other ingredients were all used raw. These ingredients were ground in a mixer as a backing material for extracts, added 100kg natural muscovado sugar and about 80L natural water (spring water in the area of Yoshi River) and natural fermented / aged at pH5 to 5.5 at the beginning. Although the period of fermentation/ maturation needed about more than 2 years, the product volume at the end was about 210kg.

2. Sample culture media and numerical count / isolation of lactic acid bacteria

1) BCP added plate count agar culture media

For isolation culture media of lactic acid bacteria, we used Agar Nissui, BCP added plate count agar culture media for measurement of lactic acid bacterial count (Nissui Pharmaceutial Co., Ltd.).

2) Modified ELLIKER culture media (*8*)

For passage and test culture media of lactic acid bacteria, we used modified ELLIKER culture media. The media structure was with 20g trypton, 5g yeast extract, 5g glucose, 4g sodium chloride, 1.5g acetic acid sodium and 0.5g L-ascorbic acid sodium. Then added purified water to make to a total volume of 1000mL (pH6.8).

3) Litmus milk

For preservation of lactic acid bacteria, we used litmus milk. The structure was 100g skim milk, 10g glucose, 1g TWEEN80, 0.1g L-cysteine· HCl · H_2O and 3g litmus. They were dissolved into distilled water to make a final volume 1000mℓ.

4) Numerical count of lactic acid bacteria and isolation method

Dilution sample was prepared 10-fold gradually from 1 mℓ fermented extract liquid which was fermented and aged for 2 years or more. Along with measuring lactic bacterial acid bacterial count after 48-hour-culture at 34℃±1℃ as plate of BCP added plate count agar culture media, we isolated its representative colony.

3. Identification Test

For identification test of lactic acid bacteria, referring to the literature of Nakae and Miyamoto (*9*) and Hasegawa (*10*), we conducted the items shown below.

1) Gram stain and bacterial morphology

With HUKER's modification (*11*), we judged according to the normal method.

2) Oxygen demand

After sterilizing BCP added plate agar high layer culture media, we inoculated piercing preserved strains. After culturing at 34℃±1℃ for 3 days, we stated those grow only on the surface part as aerobic bacteria, those grow from upper part to the middle part as facultative anaerobic bacteria and those grow only in the bottom part as strictly anaerobic bacteria.

3) Catalase test

Colonies produced on BCP added plate was collected with a platinum loop, placed the strain into small test tubes and tested whether gas would be produced or not.

4) Change in litmus milk

After culturing for 72 hours the most isolated lactic acid bacteria with litmus milk, we observed reduction, red-changed and coagulation in culture.

5) Growth temperature

From litmus milk for preservation, we replaced into modified ELLIKER culture media and tested the growth situation after the culture for 7 days under the environment not only at 34℃±1℃, 10 ℃ and 45℃.

6) NaCl resistance

On modified ELLIKER culture media, we tested its resistance after adding 6.5% to 8.0% NaCl. Above all, cultivating 1% to 10% NaCl, also concentration of 12%, 14%, 16%, 18% and 20%, we observed growth activity under these conditions.

7) The pH resistance

We inoculated sample strains to modified ELLIKER culture media prepared at pH 9.6 and observed the growth activity after 7-day-cultivation.

8) NH_3 production from arginine

We inoculated sample strains to modified ELLIKER culture media 0.3% L-arginine hydrochloride, cultured at 34℃±1℃ for 7 days, added 1mL NESSLER reagent to 4mL culture media and found that precipitated brown as positive.

Above eight items were applied to all strains of lactic acid bacteria isolated. Tests below were done upon selecting sample strains.

9) O-F test (HUGH-LEIFSON test)

On O-F test which observes "oxidation" producing acid aerobically and "fermentation" producing acid anaerobically by dissolving glucose, we added 1% volume of glucose to basic culture media (2g pepton or trypton, 5g NaCl, 0.3g K_2HPO_4, 2g agar, 0.08g BCP and 1000mL distilled water at pH7.1) and sterilized. After 48-hour culture at 34℃±1℃, we conducted the judgment.

10) Heat-tolerance for 30 minutes at 63℃

After heating modified ELLIKER culture media which inoculated sample strains, we cultured it for 5 days at 34℃±1℃ and observed the situation whether sample strains would grow or not.

11) Liquefaction of gelatin

After cultivating sample strains on broth gelatin culture media fractional sterilized (10g meat extract, 10g peptone, 5g NaCl, 150g gelatin and 1000mL distilled water at pH7.2 to 7.4) for 7 days at 25℃, we observed if the gelatin would liquefy or not.

12) Reduction of nitrate

After cultivating 0.1% nitrate potassium added culture media 10mL to modified ELLIKER culture media for 48 hours at 34℃±1℃, we added the first solution (8g sulfonyl acid dissolved in 1000mL 5N acetic acid) and the second solution (5g α-naphthyl amine dissolved in 1000mL 5N acetic acid). We found those which made precipitation changing its color to pink or brown within 30 minutes positive.

13) Sorbose fermentation

Basic culture medium (10g peptone, 5g yeast extract, 5g meat extract, 1g TWEEN 80, 0.2g L-cysteine·HCl·H_2O, 1.5g agar and 1000mL purified water at pH7.1) with BCP indicator was sterilized beforehand. Then to this added 1mL (after applied, 2% sorbose) filtrated and sterilized solution with 20% volume sorbose was applied sterilized. In this case we contrasted culture media excluding glocose. After inoculated and cultured, we judged fermentation by the yellowing of BCP.

4. Acidity of cultivating skim milk

900mL purified water was added to 100g skim milk, after sterilized for 20 minutes at 110℃, inoculated sample strains for 8 hours, 24 hours and 5 days for at 20℃ and 34℃±1℃. 10g sample was then diluted to 40mL distilled water and acidity was calculated on 1% phenolphthalein alcoholic solution as indicator by titrating with 0.1N NaOH solution.

Table 1 Experimental results for isolation and identification of lactic acid bacteria from naturally fermented extract

Strain No.	Gram strain	Morphology	Growth under condition of		
			Strict aerobic	Facultative anaerobic	Strict anaerobic
1	+	S & C	−	+	−
2	+	S & C	−	⧺	±
3	+	S & C	−	+	−
4	+	S & C	−	⧺	−
5	+	S & C	−	+	±
6	+	S & C	−	+	−
7	+	S & C	−	⧺	−
8	+	S & C	−	+	±
9	+	S & C	−	+	−
10	+	S & C	−	⧺	−
11	+	S & C	−	⧺	−
12	+	S & C	−	+	±
13	+	S & C	−	+	−
14	+	S & C	−	+	±
15	+	S & C	−	⧺	−
16	+	S & C	−	+	−
17	+	S & C	−	⧺	±
18	+	S & C	−	⧺	−
19	+	S & C	−	+	−
20	+	S & C	−	+	−
21	+	S & C	−	+	−
22	+	S & C	−	+	−
23	+	S & C	−	⧺	±
24	+	S & C	−	+	−
25	+	S & C	−	+	−

(S & C) : Spherical and chain form.
(±) : Slight growth.
(+) : Appreciable growth.
(⧺) : Significant growth.

RESULTS

1. Isolation test results of lactic acid bacteria

On preparation of fermentation extracts solution, pH was adjusted from 5.0 to 5.5 at the beginning of fermentation and maturation. The pH of the last stage of the product was 4.3. Bacterial count of culture media for isolation of lactic acid bacteria was 336 on 10^5-fold dilution plate, 52 on 10^6-fold dilution plate and 11 colonies on 10^7-fold dilution plate. Then we selected to use 10^6-fold dilution plate in this study. The colonies were picked and inoculated respectively to modified ELLIKER culture media. The culture solution was then replaced again to BCP added plate agar culture media for plate cultivation. Isolated lactic acid bacteria were then selected. As a result, on isolation test for three times from yellowed culture media and morphology of colonies, a total number of 25 strains were selected as isolated lactic acid bacteria No.1 to No.25. These isolated strains were then inoculated on litmus milk and preserved.

Table 2 Experimental results for isolation and identification of lactic acid bacteria from naturally fermented extract

Strain No.	Catalase test	Change in litmus milk		
		Reduction	Acid production	Coagulation
1	−~±	+	+	+
2	+~±	+	++	+
3±	+	++	+	
4	−~±	+	+	+
5	−~±	+	++	+
6	+~±	+	+	+
7	−	+	+	+
8	±	+	++	+
9	+~±	+	++	+
10	+~±	+	++	+
11	−~±	+	+	+
12	−~±	+	+	+
13	±	+	++	+
14	−~±	+	+	+
15	±	+	+	+
16	+~±	+	++	+
17	−~±	+	+	+
18	−	+	++	+
19	+~±	+	+	+
20	−~±	+	++	+
21	+~±	+	++	+
22	−~±	+	++	+
23	−~±	+	+	+
24	−~±	+	++	+
25	−	+	++	+

(−) : No change.
(±) : Slight change.
(+) : Appreciable change.
(++) : Significant change.

Table 3 Experimental results for isolation and identification of lactic acid bacteria from naturally fermented extract

Strain No.	Growth at		Growth at NaCl 6.5%	Growth at pH 9.6	NH_3 from arginine
	10℃	45℃			
1	+	−	++	++	+*
2	−	−	++	+	+
3	+	−	+++	++	+
4	−	−	++	++	+
5	+	−	++	+	+
6	+	−	+	+	+
7	+	−	++	+	+
8	−	−	+++	++	+
9	+	−	++	++	+
10	−	−	++	+	+
11	+	−	++	+	+
12	+	−	++	++	+
13	+	−	++	++	+
14	+	−	+++	++	+
15	+	−	++	+	+
16	+	−	++	+	+
17	+	−	++	++	+
18	+	−	++	++	+
19	−	−	+++	++	+
20	−	−	++	+	+
21	−	−	++	+	+
22	+	−	++	+	+
23	+	−	++	++	+
24	−	−	+++	++	+
25	+	−	++	+	+

(−) : No growth.
(+) : Appreciable growth.
(++) : Moderate growth.
(+++) : Significant growth.
(*) : Positive in NH_3 production.

2. Identification test results

On the previous isolated and selected strains, Table 1 shows test results of Gram stain, morphology and oxygen demand and Table 2 shows test results of catalase test and litmus milk change. The result of Gram stain test shows all Gram positive and spherical/chain form. On oxygen demand test, No. 5 and No. 12 had their bottom part yellowed while the rest strains had their middle and upper part yellowed. All strains were judged anaearobic. On catalase test, most strains were negative or quasi-positive (- to ± or ±) while strains No. 2, 6, 9, 10, 16, 19 and 21 were quasi-positive or positive. On litmus milk change, within three days of inoculation all strains had reduction, change in red and coagulation. Each strain showed their own changing rate.

Table 3 shows growth of sample strains at 10℃ and 45℃, resistance of 6.5% NaCl, pH9.6 resistance and test result of NH_3 production from arginine.

Table 4 Experimental results for isolation and identification of lactic acid bacteria from naturally fermented extract

Strain No.	OF-test		Tolerance at 63℃ for 30 minutes	Liquifaction of gelatin	Nitrate reduction	Acid from sorbose
	Oxidation	Fermentation				
2	−	+	+	−	−~±*	−
7	−	+	+	−	−~±	−
13	−	+	+	±	±	+
23	−	+	+	−	−~±	−

(−): Negative change. *(−): No change.
(+): Positive change. (±): Slight change.
(±): Slight change. (+): Appreciable change.

Table 5 Growth Tolerance in various concentration of NaCl

Strain No.	Concentration of NaCl (%)														
	1	2	3	4	5	6	7	8	9	10	12	14	16	18	20
2	⋕	⋕	⋕	⋕	⋕	⋕	⋕	+	+	+	+	+	+	+	−
7	⋕	⋕	⋕	⋕	⋕	⋕	⋕	+	+	+	+	+	+	−	−
13	⋕	⋕	⋕	⋕	⋕	⋕	⋕	+	+	+	+	+	+	±	±
23	⋕	⋕	⋕	⋕	⋕	⋕	⋕	+	+	+	+	+	+	+	+

(−): No growth.
(±): Slight growth.
(+): Appreciable growth.
(⋕): Significant growth.
(⋕): Rapid and strong growth.

Table 6 Acid production during incubation at 20℃ and 34℃ in skim milk

Strain No.	Incubation at 20℃ for:			
	8 hr	24 hr	48 hr	5 days
2	0.27	0.28	0.28	0.30
7	0.26	0.27	0.28	0.31
13	0.27	0.28	0.30	0.30
23	0.27	0.28	0.30	0.30
Strain No.	Incubation at 34℃±1℃ for:			
	8 hr	24 hr	48 hr	5 days
2	0.25	0.36	0.42	0.49
7	0.25	0.37	0.46	0.56
13	0.23	0.39	0.43	0.52
23	0.26	0.38	0.46	0.51

On results of growth test at 10℃ and 45℃, after 7-day cultivation, 17 strains out of 25 strains grew at 10℃ while 8 strains did not grow. On the growth test at 45℃, no strain admitted the growth. Against 6.5% NaCl, all strains showed resistance. Especially, 5 strains as No.3, 8, 14, 19 and 24 had extreme growth and high resistance. Also on pH 9.6 resistance, all 25 strains showed full growth after 7-day cultivation. Moreover, since this strainshowed pH4.3 on fermentation extract at the beginning, this strain was judged inhabitable in a wide range from pH4.3 to 9.6. Also, on NH_3 production from arginine, all 25 strains showed positive. From all above test results, we can infer that these strain belong to the genus *Streptococcus* of *Enterococcus* group.

3. Selection of isolated strains and other test results

As a result of more than 25 strains identification test, those with similarity were gathered and categorized in four groups: 1) those of (±) on catalase test, 2) those of (- to ±), 3) those of (-) and 4) those of (+ to ±).

We selected 4 strains as representative strains as No.2, 7, 13 and 23. With these 4 strains, we conducted O-F test, heat-tolerance for 30 minutes at 63℃, liquefaction of gelatin, reduction of nitrate and acid production test from sorbose. Table 4 shows the results.

On O-F test, they were anaerobic and fermentative. On heat-tolerance test for 30 minutes at 63℃, all

strains grew and showed heat-tolerance activity. On gelatin liquefaction test, three strains were negative and 1 strain was quasi-positive (±). On nitrate reduction test, three strains were negative or quasi-positive (- to ±) and 1 strain was quasi-positive (±). On fermentation test from sorbose, three strains were negative and one strain was positive. Table 5 shows 1% to 20% NaCl resistance and its test results. NaCl resistance was found in an extremely broad range. Active range was shown at 1% NaCl to 7% NaCl. Results of acid production test on skim milk culativation are shown in Table 6. After 5-day cultivation at 20℃, acidity increase was extremely low as 0.30 to 0.31%. At 34℃±1 ℃, the value shown was 0.49% to 0.56%.

STUDY

This isolated strain were anaerobic; many of these showed negative and quasi-positive in catalase test; had litmus milk reduction, change in red and coagulation; and showed growth at 10℃. They also showed resistance in 6.5% NaCl and full growth at pH9.6 while NH_3 production from arginine.

On O-F test, anaerobic and fermentative activities were confirmed; and growth was shown under heat-tolerance for 30 minutes at 63℃. On gelatin liquefaction test, one strain showed quasi-positive while three strains showed negative. On nitrate reduction test, one strain showed quasi-positive while three strains showed negative.

Notably, these isolated strains were extremely high in salt-tolerance and inhabitable at 18% to 20% NaCl. According to Bergey's 8th, Determinative Bacteriology, *Enterococcus* group is considered inhabitable up to about 6.5% NaCl. However, as seen in Ito's research (*12*), it is approved that they are fully inhabitable at 15% NaCl in a certain condition of growth environment or culture medium. Stronger salt-tolerance was recognized in these isolated strains than the value in Ito's literature (*12*). According to the results of isolation/identification test, the difference between the genus *Streptococcus* and *Enterococcus* group had no growth at 45℃. Although strains similar to *Enterococcus* group which do not grow at 45℃ are *Streptococcus lactis*, they are considered not be equivalent to *Streptococcus lactis* judging from the series of identification test results.

Also, the cause of no growth at 45℃ can be inferred because the culture media of fermentative extract of this isolated strain lead fermentation and maturation under relatively low temperatured natural environment in highlands. With the results all above, the most similar strains in its characteristics in the genus *Streptococus* are *Streptococcus faecalis* and *Streptococcus faecium*. Also, the difference between both strains is sorbose fementation. From identification test here, selected 4 strains of lactic acid bacteria are 3 strains of *Streptococcus faecalis* and one strain of *Streptococcus faecium*.

SUMMARY

This research was carried out to isolate and identify especially lactic acid bacteria from fermented extract naturally fermented and aged over 2 years in highlands by selecting traditionally eaten wild terrestrial and aquatic plants (4 species) and wild fruits (7 species). The ingredients for fermentation such as Chinese bayberry (*Myrica rubra*), wild vine (*Vitis coignetiae*), akebi (*Akebia quinata*), mulberry (*Morus australis*), oleaster (*Elaeagnus montana*), Chinese matrimony (*Lycium chinese*), broad-leaved plantain (*Plantago asiatica*), mugwort (*Artemisia montana*), brown algae (*Hijikia fushiforme*), and sea tangle (*Laminaria japonica*) those row materials are ground in a mixer, those dry materials are also ground in a mixer after wetted, Chinese matrimony was used in powder and mixed with natural muscavado for fermention and maturation under natural environment. Bacterial count of live lactic acid bacteria from fermentation extract solution was 5.2×10^7 /mL. From this test, wild strains *Streptococcus faecalis* and *Streptococcus faecium* were isolated which showed high salt-tolerance and were unable to grow at 45℃.

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