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I. Original Reports
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2. Original Reports

Biochemical Properties of Lactic Acid Bacteria from Traditional Fermented Foods in Southeast Asia

1. Salt-, Acid- and Heat-Tolerance and Acid-Production of Lactic Acid Bacteria from Traditional Fermented Foods in Southeast Asia

Iichiroh Ohhira, Jung Chang Min, Taku Miyamoto and Kei Kataoka
Division of Applied Production Technology, Graduate School of Natural Science and Technology, Okayama University, Okayama, 700 Japan

Summary

From traditional fermented foods in Southeast Asia, 4 genuses, 15 species, 189 strains of lactic acid bacteria were isolated and identified. Fourty-six strains of lactic acid bacteria were examined salt-, acid - and heat-tolerances and acid production activities.

Pediococcus halophillus, and *Leuconostoc paramesenteroides* could be grown in 10% NaCl added broth medium, *Lactobacillus casei* subsp. *pseudoplantarum, Ped. halophilus* and *Leuconostoc lactis* were able to be grown in pH 3.5 and *Ped. halophilus, Leuc. mesenteroides* and *Streptococcus faecalis* had been active at 45℃.

Acid production activities of *Str. bovis, Str. faecalis, Str. faecium, Leuc. mesenteroides* were high and *Str. bovis* which showed the highest activity produced 0.42% lactic acid after 24 hours in broth media.

Selecting strains with excellent properties by screening biochemical properties of lactic acid bacteria in their functional characteristics in cultivation and metabolism is beneficial for food processing.

In such point of view, following studies have been done: screening excellent strains such as salt-tolerance lactic acid bacteria (*1*), or acid-tolerance lactic acid bacteria (*2*), furthermore, acid-production of lactic acid bacteria as metabolic function of lactic acid bacteria (*2*), proteolysis (*3*), fat dissolution (*4*), aroma-production (*5*), anti-bacteria production (*6,7*) and others. Also, in aim to expand the usage of lactic acid bacteria, it is believed to need the screeing of special saccharide fermentation or heat-tolerance lactic acid bacteria. These studies would have functional characteristic each lactic acid bacteria improve the preservation of fermented foods so that it is important in adding flavor or taste.

In the meantime, Southeast Asia belongs to the Tropical Zone geographically and obtains a variety of mainly salted traditional fermented foods due to their climate peculiarity. In the natural fermented foods born under these peculiar environment, lactic acid bacteria with especial strong fermentation activity are estimated to exist.

Thus, we tested lactic acid bacteria isolated from traditional fermented foods in Southeast Asia and examined on its properties of cultivation and metabolism in our previous reports (*8,9,10*). For properties of cultivation, we researched on salt-, acid- and heat-tolerance. And for properties of metabolism, we researched on acid-production, proteolysis and aroma-production. Then we studied on its benefit by screening lactic acid bacteria strains with excellent properties.

I. Salt-, Acid-, and Heat-Tolerance of Lactic Acid Bacteria Isolates

Screening lactic acid bacteria which are able to grow in peculiar environment and selecting lactic acid bacteria which are able to apply for many kinds of fermentation ingredients are important in aim to widen the usage of lactic acid bacteria.

For example, on food fermentation under high constituency salt content, if lactic acid bacteria containing salt-tolerance exists, it would satisfy the purpose of preservation, aroma-production and taste, and have an ideal and important meaning on food production. Also, if acid-tolerance lactic acid bacteria are selected, it is beneficial for fermented foods preservation. We could think of the possibilities applying to enterobacterial flora relating to human health.

On the other hand, screening heat-tolerance lactic acid bacteria would inhibit the growth of low temperature harmful microbes. The usage of such enzyme is considered beneficial in developing new food ingredients.

With above points of view, we experimented in aim to screen lactic acid bacteria with salt-, acid-, and heat-tolerance from 46 strains of representative isolated lactic acid bacteria.

METHOD

1) Test samples

In our previous reports (*8, 9, 10*), 189 strains isolated from 16 samples of traditional fermented foods of Southeast Asia were isolated into 4 genera and 15 species. Among those, we selected representative strains and tested 46 strains in total. On selecting the strains, we considered the source of isolation, the ratio of strains isolated by each document, and characteristic of the toxology.

Table 1 shows its details. That is, we selected the following strains: 2 strains from coconuts wine and 1 strain from *s. belachan* identified as *L. casei* subsp. *casei* of the genus *Latobacillus,* 1 strain from *idli* and 2 strains from *tape* identified as *L. casei* subsp. *pseudoplantarum,* 1 strain from s. belachan identified as *L. casei* subsp. *rhamnosus,* 1 strain from rice wine , 1 strain from *dosai* and 1 strain from *tauco* identified as *L. coryniformis* subsp. *coryniformis,* and 1 strain from *dadih,* 2 strains from *belachan,* 3 strains from *budu,* 1 strain from *sambal belachan* and 2 strains from *tempoyak* identified as *L. plantarum.* From the genus *Streptococcus,* we selected 1 strain from dosai identified as *Str. bovis,* 2 strains from *tempeh,* 1 strain from s. belachan and 1 strain from tempoyak identified as *Str. faecalis,* 1 strain from *cinchaluk,* 1 strain from *kicap,* 1 strain from sambal belachan, and 1 strain from *trassi* identified as *Str. faecium,* 1 strain from sambal belachan identified as *Str. gallinarum,* and 2 strains from dadih identified as *Str. lactis.*

Also, in the genus *Leuconostoc,* we selected 1 strain from belachan, 1 strain from cinchaluk, 1 strain from pekasan, 2 strains from tauco, 2 strains from trassi, and 1 strain from tempoyak identified as *Leuc. mesenteroides* subsp. *mesenteroides,* 1 strain from dadih identified as *Leuc. lactis,* and 1 strain from kicap identified as *Leuc. paramesenteroides.*

On the other hand, from the genus *Pediococcus,* we selected 2 strains from idli identified as *Ped. halophilus* and 4 strains identified as *Ped. pentosaceus.* We tested 4 genera, 15 species, and 46 strains in total.

2) Salt-tolerance test

Sodium chloride was added to modified Elliker culture broth, each of them were prepared to 5%, 10%, 15% and 20% in constituency. After these culture broths were filtrated and sterilized. We inoculated sample strains and cultivated for 10 days at 34℃. We observed if they grew or not.

Table 1 Strains used for biochemical properties of lactic acid bacteria isolated from traditional fermented foods in Southeast Asia

Species	Source	Strain No.
L. casei subsp. *casei*	Coconut wine	3
	Coconut wine	9
	S. Belachan	2
L. casei subsp. *pseudoplantarum*	Idli	13
	Tape	1
	Tape	15
L. casei subsp. *rhamnosus*	S. Belachan	3
L. coryniformis subsp. *coryniformis*	Rice wine	13
	Dosai	7
	Tauco	14
L. plantarum	Dadih	14
	Belachan	7
	Belachan	10
	Budu	1
	Budu	3
	Budu	15
	S. Belachan	14
	Tempoyak	7
	Tempoyak	14
Leuc. mesenteroides subsp. *mesenteroides*	Belachan	1
	Cinchaluk	13
	Pekasam	1
	Tauco	8
	Tauco	9
	Trassi	1
	Tempoyak	5
Leuc. paramesenteroides	Kicap	1
Leuc. lactis	Dadih	15
Str. bovis	Dosai	10
Str. faecalis	Tempeh	10
	Tempeh	15
	S. Belachan	10
	Tempoyak	8
Str. faecium	Cinchaluk	1
	Kicap	13
	S. Belachan	9
	Trassi	10
Str. gallinarum	S. Belachan	5
Str. lactis	Dadih	1
	Dadih	9
Ped. halophilus	Idli	3
	Idli	7
Ped. pentosaceus	Rice wine	2
	Rice wine	6
	Rice wine	14
	Rice wine	15

Table 2 Salt-tolerance of selective lactic acid bacteria isolated from traditional fermented foods in Southeast Asia

Species	Growth in NaCl				Species	Growth in NaCl			
	5%	10%	15%	20%		5%	10%	15%	20%
L. casei subsp. *casei*	+	−	−	−	*Leuc. mesenteroides* subsp. *mesenteroides*	+	−	−	−
L. casei subsp. *pseudoplantarum*	+	−	−	−	*Leuc. paramesenteroides*	+	+	−	−
L. casei subsp. *rhamnosus*	+	−	−	−	*Leuc. lactis*	+	−	−	−
L. coryniformis subsp. *coryniformis*	+	−	−	−	*Str. faecalis*	+	+/−	−	−
L. plantarum	+	−	−	−	*Str. faecium*	+	+/−	−	−
Ped. halophilus	+	+	−	−	*Str. gallinarum*	+	−	−	−
Ped. pentosaceus	+	−	−	−	*Str. lactis*	+	−	−	−
					Str. bovis	+	−	−	−

Table 3 Heat-tolerance and acid-tolerance of selective lactic acid bacteria isolated from traditional fermented foods in Southeast Asia

Species	Growth at				Growth at pH			
	40°C	45°C	50°C	55°C	3.0	3.5	4.0	4.5
L. casei subsp. *casei*	+	−	−	−	−	−	+	+
L. casei subsp. *pseudoplantarum*	+	−	−	−	−	+	+	+
L. casei subsp. *rhamnosus*	+	−	−	−	−	−	+	+
L. coryniformis subsp. *coryniformis*	+	−	−	−	−	−	+	+
L. plantarum	+	+/−	−	−	−	+/−	+/−	+
Leuc. mesenteroides subsp. *mesenteroides*	+	+	−	−	−	−	−	−
Leuc. paramesenteroides	+	+	−	−	−	−	−	−
Leuc. lactis	+	−	−	−	−	−	−	−
Str. faecalis	+	+/−	−	−	−	+/−	+/−	+
Str. faecium	+	+	−	−	−	−	+/−	+
Str. gallinarum	+	−	−	−	−	−	+	+
Str. lactis	−	−	−	−	−	−	+	+
Str. bovis	+	−	−	−	−	−	+	+
Ped. halophilus	+	+	−	−	−	+	+	+
Ped. pentosaceus	+	−	−	−	−	−	+	+

3) Acid-tolerance test

10% lactic acid was added to modified Elliker culture broth, prepared to become each with pH3.0, pH3.5, pH4.0 and pH4.5. After filtrated and sterilized, we inoculated sample strains, and cultivated for 10 days at 34℃. Thus, we observed if they grew or not.

4) Heat-tolerance test

Each of the test samples were inoculated to modified Elliker culture broth. We cultivated for 10 days at 40℃, 45℃, 50℃, and 55℃ and observed their growth.

Table 4 pH values, salt concentration and lactic acid bacterial counts of traditional fermented foods in Southeast Asia and number of isolates

Samples used	pH	% NaCl	Bacterial counts		Number of isolates
			BCP[2]	MRS[2]	
Alcoholic drink					
Coconut wine	3.79	0.29	3.0×10^{7}	4.2×10^{7}	3
Rice wine	4.35	0.36	1.8×10^{6}	6.0×10^{5}	10
Side dish food					
Dadih	4.29	0.16	3.7×10^{8}	3.8×10^{8}	9
Dosai	3.62	0.08	6.0×10^{7}	5.0×10^{7}	6
Idli	4.32	0.66	2.4×10^{10}	2.1×10^{8}	8
Tape	4.75	0.01	2.4×10^{8}	3.6×10^{8}	10
Source food					
Belachan	8.75	1.77	6.1×10^{8}	3.5×10^{8}	15
Budu	5.02	8.66	2.0×10^{6}	2.0×10^{7}	15
Cincaluk	5.72	4.65	7.0×10^{6}	2.0×10^{7}	15
Kicap	5.93	5.82	3.0×10^{7}	9.0×10^{7}	15
Pekasam	5.67	1.51	1.9×10^{9}	6.7×10^{8}	15
Sambal belachan	4.25	0.32	1.3×10^{10}	6.9×10^{8}	15
Tauco	5.74	1.34	2.0×10^{6}	3.0×10^{5}	15
Tempoyak	4.15	0.12	9.0×10^{7}	1.6×10^{8}	15
Trassi	7.25	2.48	4.0×10^{6}	5.0×10^{6}	15

1) Salt concentration was estimated by using Sinar salt meter (NS-3P).
2) BCP added plate count agar and MRS agar were used.

RESULTS AND STUDY

Table 2 and Table 3 show the results on salt-, acid-, and heat-tolerance of isolated lactic acid bacteria.

In salt-tolerance test on 5% sodium chloride content culture broth, vigorous growth on every strain was observed. However, on 10% sodium chloride content culture broth, growth was admitted only on strains identifed as *Ped. halophilus* and *Leuc. paramenteroides,* and on a part of strains of *Str. faecalis* and *Str. faecium.* Growth on 15% sodium chloride content culture broth and 20% sodium chloride content culture broth was not admitted in any strain. Although Bergey's new edition (*11*) writes that *Ped. halophilus* is able to grow on 15% sodium chloride content culture broth, we could not find any growth on 15% sodium chloride content culture broth in this research. We estimate that salt-tolerance decreased because they grew under the environment with less salt content (Table 4) in the sample of idli as the source of isolate.

In result of studying acid-tolerance, test sample of pH3.0 was able to grow. On pH3.5 culture broth, the full growth was seen in *L. casei* subsp. *pseudoplantarum, Ped. halophilus* and *Leuc. lactis.* A part of *L. plantarum* and *Str. faecalis* also showed the growth. However, on other strains, their growth was not observed.

Under the condition of pH4.0, strains *Leuc. mesenteroides* subsp. *mesenteroides* and *Leuc. paramesenteroids* could not grow, and strains *L. plantarum, Str. faecalis* and *Str. faecium* showed both possibilities and impossibilities of growth. In other strains, any of them showed growth.

On above results, acid-tolerance of lactic acid bacteria is assumed to be around pH3.0 as its limit zone.

In heat-tolerance test, the growth in all strains expect for *Str. lactis* at 40℃ was admitted. In the growth test at 45℃, the growth was seen in *Ped. halophilus, Leuc. mesenteroides* subsp. *mesenteroides, Leuc. paramensenteroides,* and *Str. faecium.* However, we found out that in strains identified as *L. plantarum* and *Str. faecalis* there were strains that are able to grow and those that are not able to grow. Also, at 50℃ and 55℃, no growth was seen on any test samples.

As high heat-tolerance strains, we picked *L. delbrueckii, L. helveticus, Str. thermophilus* and *Ped. acidilactici* which were mentioned in Bergey's new version (*11*) as being able to grow at 50℃. However, these strains were not included in this research, its limit of heat-tolerance was around 45℃.

SUMMARY

We experimented salt-, acid-, and heat-tolerance test on each strain on 46 selected strains.

On salt-tolerance test, those admitted the growth on 10% sodium chloride content culture broth were *Ped. halphilus* and *Leuc. paramesenteroides. Str. faecalis* was isolated including those with and without tolerance although they are the same species.

On acid-tolerance test, in any test strains, no growth was admitted on pH3.0 but the growth of *L. casei* subsp. *pseudoplantarum, Ped. halophilus* and *Leuc. lactis.* In strains L. plantarum and Str. faecalis, there were strains able and unable to grow at pH3.5.

On heat-tolerance test, those strains able to grow at 45℃ were *Ped. halophilus, Leuc. mesenteroides* subsp. *mesenteroides, Leuc. paramesenteroides, Str. faecium* and the same species of strain, but isolated strains identified as *L. plantarum* and *Str. faecalis* including partly unable to grow. We could not find any strains able to grow above 50℃ as we expected.

Ⅱ. Acid production of isolate lactic acid bacteria

On acid production of lactic acid bacteria, the speed of acid production and the total volume of acid production are on issue. The faster the strain in acid production speed cannot be the greater in total acid production volume. For example, in considering the usage to milk products, the former has significance in usage as lactic acid production or starter as cheese in part. With these points of view, we examined on acid production of lactic acid bacteria in skim milk culture medium in this research and screened superior strains.

METHODS

1) Sample strains

We used 46 strains, representative isolate strains as sample strains as in previous section.

2) Sample culture medium

For acid production test culture medium, we used skim milk culture medium. The formation of the culture medium is as follows: purified water is added to 5g glucose and 100g skim milk to be 1000mL in total. We also prepared the same formation of culture medium containing 5% sodium chloride.

3) Measurement of acid production

We searched on acid production in aim to study the outline of acid production speed and total acid production volume of each test strain as in the following method.

That is, we used 10ml of above skim milk culture media (0.14-0.15 acidity) as culture radical and heated to cultivation temperature 34℃ beforehand. Then we inoculated a platinum loop of 24-hour culture solution of the same skim milk culture medium and measured lactic acid acidity of acid production along with measuring the pH value of 24 hours after the beginning of the cultivation.

Table 5 Acid production in skim milk with and without sodium chloride of lactic acid bacteria isolated from traditional fermented foods in Southeast Asia

Species	Source	Strain No.	Acidity*		pH value	
			o% NaCl	5% NaCl	0% NaCl	5% NaCl
L. casei subsp. *casei*	Coconut wine	3	0.21	0.22	5.34	5.50
	Coconut wine	9	0.33	0.22	5.56	6.02
	S. Belachan	2	0.24	0.20	6.13	5.84
L. coryniformis subsp. *coryniformis*	Rice wine	13	0.19	0.17	6.33	6.13
	Dosai	7	0.20	0.21	6.28	6.02
	Tauco	14	0.16	0.17	6.36	6.14
L. casei subsp. *pseudoplantarum*	Idli	13	0.19	0.17	6.30	6.19
	Tape	1	0.17	0.17	6.33	6.15
	Tape	15	0.20	0.17	6.18	6.12
L. casei subsp. *rhamnosus*	S. Belachan	3	0.34	0.27	5.47	5.84
L. plantarum	Dadih	14	0.34	0.18	6.22	6.17
	Belachan	7	0.21	0.21	6.24	6.00
	Belachan	10	0.19	0.20	6.17	6.13
	Budu	1	0.19	0.17	6.24	6.22
	Budu	3	0.19	0.17	6.24	6.13
	Budu	15	0.26	0.16	6.10	6.22
	S. Belachan	14	0.24	0.20	5.94	5.97
	Tempoyak	7	0.24	0.15	6.02	6.22
	Tempoyak	14	0.24	0.15	6.07	6.23

* Control values were 0.14% and 0.16% in skim milk with and without sodium chloride, respectively.

RESULTS AND STUDY

Table 5 - 8 show the results of acidity of production after 24-hour cultivation of test strains and the pH value of culture medium.

We measured every strain both in culture media with and without containing sodium chloride. As we put on the footnote of Table 5 - 8, we studied acid production of each test lactic acid strains from the results of acidity production since the acidity of standard culture media without strains and the pH value were almost same.

First, the strain with the most volume of acid production in skim milk culture medium without sodium chloride was *Str. bovis* (dosai No.10) isolated from dosai with 0.42% at 24th hour. Looking at each species, the volume of acid production of *Str. faecalis* showed a high tendency and *L. plantarum* was low species (Fig.1). Also in the report of Sasaki *et.al.* (*2*), the acid production of *Str. faecalis* show superior. The strains with high in acid production volume on 5% sodium chloride content skim milk culture medium were *Leuc. mesenteroides* subsp. *mesenteroides* and *Leuc. paramesenteroides*, and *Str. faecium*. The low strains that were *L. plantarum*, *Str. lactis* and *Ped. halophilus*.

From the above results, *Str. bovis* (dosai No.10), *Str. faecalis* (tempeh No.15, tempoyak No.8), *Str. faecium* (trassi No.10), *Leuc. mesenteroides* subsp. *mesenteroides* (trassi No.1, tempoyak No.5), *Leuc. paramesenteroides* (kicap No.1) showed high in acid production volume in culture media without sodium chloride, but in 5% sodium chloride content culture media, the volume of acid production showed an extremely decreasing tendency. However, in the study of the previous section, since the above strains are able to grow under the existence of 5% sodium chloride, the decrease of acid production is estimated to result from the decrease of acid production activity by the strain of sodium chloride.

Table 6 Acid production in skim milk with and without sodium chlorde of lactic acid bacteria isolated from traditional fermented foods in Southeast Asia

Species	Source	Strain No.	Acidity*		pH value	
			0% NaCl	5% NaCl	0% NaCl	5% NaCl
Str. bovis	Dosai	10	0.58	0.18	4.74	6.20
Str. faecalis	Tempeh	10	0.22	0.24	6.11	5.94
	Tempeh	15	0.50	0.22	5.05	5.85
	S. Belachan	10	0.36	0.20	5.87	5.90
	Tempoyak	8	0.42	0.23	5.25	5.75
Str. faecium	Cinchaluk	1	0.42	0.30	5.24	5.60
	Kicap	13	0.30	0.30	5.72	5.62
	S. Belachan	9	0.19	0.27	6.23	6.22
	Trassi	10	0.43	0.26	5.36	5.67
Str. gallinarum	S. Belachan	5	0.23	0.17	6.18	6.22
Str. lactis	Dadih	1	0.28	0.31	5.69	6.18
	Dadih	9	0.30	0.29	5.67	6.23

* Control values were 0.14% and 0.16% in skim milk with and without sodium chloride, respectively.

Table 7 Acid production in skim milk with and without sodium chloride of lactic acid bacteria isolated from tradition fermented foods in Southest Asia

Species	Source	Strain No.	Acidity*		pH value	
			0% NaCl	5% NacCl	0% NaCl	5% NaCl
Leuc. mesenteroides subsp. *mesenteroides*	Belachan	1	0.32	0.31	5.72	5.52
	Cinchaluk	13	0.28	0.32	5.73	5.50
	Pekasam	1	0.35	0.31	5.60	5.54
	Tauco	8	0.23	0.25	5.97	6.13
	Tauco	9	0.26	0.23	6.02	6.10
	Trassi	1	0.51	0.27	5.31	5.80
	Tempoyak	5	0.43	0.23	5.23	5.51
Leuc. lactis	Dadih	15	0.19	0.19	6.36	6.15
Leuc. paramesenteroides	Kicap	1	0.52	0.31	5.34	5.50

* Control values were 0.14% and 0.16% in skim milk with and without sodium chloride respectively.

Not regarding to the existence of 5% sodium chloride, strains with a tendency showing acid production averagely are named such as *Str. faecium* (Kicap No.13, S. Belachan No.9), *Leuc. mesenteroides* subsp. *mesenteroides* (Belachan No.1, Pekasam No.1) and others.

On the other hand, *Ped. halophilus* showing salt-tolerance in the study of previous section showed a relatively lower tendency in the volume of acid production compared to other species of strains. This is believed to effect the growth of skim milk culture broth we used here.

SUMMARY

Proceeding to the previous section, we researched on acid production on skim milk culture medium with 46 strains of sample lactic acid bacteria. As a result, *Str. bovis* (dosai No.10), *Str. faecalis* (tempeh No.15, tempoyak No.8), *Str. faecium* (trassi No.10), *Leuc. mesenteroides* subsp. *mesenteroides* (trassi No.1, tempoyak No.5) and *Leuc. paramesenteroides* (kicap No.1) were isolated as the strains with high in acid production volume after 24-hour cultivation.

Table 8 Acid production in skim milk with and without sodium chloride of lactic acid bacteria isolated from traditional fermented foods in Southest Asia

Species	Source	Strain No.	Acidity*		pH value	
			0% NaCl	5% NaCl	0% NaCl	5% NaCl
Ped. halophilus	Idli	3	0.17	0.18	6.35	6.19
	Idli	7	0.19	0.15	6.22	6.22
Ped. pentosaceus	Rice wine	2	0.18	0.17	6.37	6.20
	Rice wine	6	0.23	0.18	6.29	6.22
	Ricewine	14	0.20	0.16	6.38	6.22
	Rice wine	15	0.18	0.17	6.36	6.22

* Control values were 0.14% and 0.16% in skim milk with and without sodium chloride, respectively.

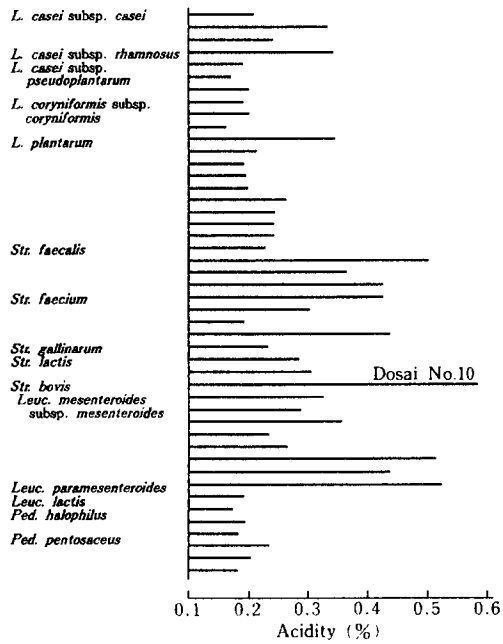

Fig. 1 Acid production of isolates.

Among them, acid production of *Str. bovis* is extremely high showing 0.42% after 24-hour cultivation. However, acid production volume of these strains in 5% sodium chloride content skim milk culture media showed generally a decreasing tendency.

Not regarding to either skim milk culture media without sodium chloride and with 5% sodium chloride, the following strains were isolated as species showing acid production and suggested to use as salted fermented foods: *Str. faecium* (kicap No.13, s. belachan No.9) and *Leuc. mesenteroides* subsp. *mesenteroides* (belachan No.1, cinchaluk No.13, pekasan No.1).

While *Ped. halophilus* as halotorelant lactic acid bacteria showed not so high in acid production volume on both culture broths. We would consider that the formation of the culture media we used have the influence.

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