Identification of Compounds Causing Symbiotic Growth of *Lactobacillus paracasei* subsp. *tolerans* and *Kluyveromyces marxianus* var. *lactis* in Chigo, Inner Mongolia, China

Satomi ISHII, Masanori KIKUCHI, Kei MURAMATSU and Syouichi TAKAO

Faculty of Dairy Science, Rakunou University, Ebetsu-shi, 069-8501, Japan

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Abstract “Chigo”, a traditionally fermented mares’ milk in Inner Mongolia, China (known as kumiss in Russia) was used in this study. We investigated microbial flora and proliferation properties using lactic acid bacteria and yeast isolated from “Chigo”. Mixed strains of the isolates were cultivated in 10% reconstituted skim milk (RSM) medium, and the combination of *Lactobacillus paracasei* subsp. *tolerans* (LB) and *Kluyveromyces marxianus* var. *lactis* (KM) was found to produce the highest quantity of lactic acid. The nutrient requirements of LB were investigated by omission test and eight amino acids (Ala, Val, Phe, Ser, Thr, Glu, Lys and Arg) and seven vitamins (thiamin, riboflavin, niacin, pantothenic acid, biotin, pyridoxine and folic acid) were found to be essential. When the above mentioned amino acids and vitamins were added to the RSM medium, glutamic acid, serine, folic acid and niacin showed highly stimulatory effect on lactic acid production followed by addition of the supernatant of the yeast culture and the cell-free extract of the yeast. It is thought that nutrients for the growth of lactic acid bacteria may have remained in the cell-free extract of the yeast. Amino acids excreted by KM were analyzed by an amino acid analyzer. Results suggest that a large amount of peptides accumulated in the mixed culture may be responsible for the higher number of viable cells and increased production of lactic acid in the mixed culture containing LB and KM, compared with the single culture of the LB.

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Lactic acid bacteria and yeast are used to produce various types of fermented foods such as pickles, refined sake, miso, soy sauce and fermented milk. A wide variety of symbiotic relationships are considered to exist among these microorganisms^{15,17)}.

Chigo, an unrefined sake-like fermented milk produced from mares’ milk through alcoholic fermentation^{1,8)} was used in this study. It is called “Chigo” or “Chige” in Inner Mongolia, China ; “Airag” in the Mongolia ; , and “Kumiss” in Russia^{6,12)}. Since foreigners have been forbidden to enter the chigo-producing areas from the World War II period, sufficient microbiological investigation of

chigo has not been performed.

Chigo is produced through the interaction between lactic acid bacteria and yeast. As an example of this type of symbiotic relationship, Hirota⁴⁾ reported a strong interaction observed between *Lactobacillus brevis* and *Lactobacillus bucheri* and *Candida pseudotropicalis*, *Saccharomyces* sp., all of which were isolated from kefir. Leroi and Pidoux^{10,11)} reported that *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from a sugary kefir grain, a traditional fermented drink in the Angers area of France, have a strong interaction when proliferated in the mixed culture. Furthermore, Leroi and Curcoux⁹⁾

Corresponding : Satomi ISHII (fax : +81 (0) 387-5848)

reported that in addition to the initial yeast to bacteria ratio and incubation temperature at the initiation of culture, pH is also an important factor that stimulates proliferation of lactic acid bacteria in the mixed culture.

Considering the possibility of the existence of a commensal relationship between lactic acid bacteria and yeast which are involved in the fermentation of chigo, we isolated and identified strains of lactic acid bacteria and yeast from chigo. As described in the previous report, the strains of lactic acid bacteria isolated from chigo were *Lactobacillus rhamnosus*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus paracasei* subsp. *tolerans* and *Lactobacillus curvatus*, and as a strain of yeast, *Kluyveromyces marxianus* var. *lactis* was isolated⁵⁾.

The authors reported that the combination of *Lactobacillus paracasei* subsp. *tolerans* (LB) and *Kluyveromyces marxianus* var. *lactis* (KM) had the most significant effects on lactic acid production. In this study, we evaluated the number of viable cells, lactic acid production and ethanol production in the single or mixed cultures of the selected combination of LB and KM. Amino acids and vitamins requirements of LB were confirmed. In addition, by adding culture medium of yeast, cell-free extract of yeast and heating them to produce the medium for single culture, we also examined whether LB utilizes the substances produced by KM on the basis of lactic acid production. Finally, the amounts of free amino acids and peptides in single or mixed cultures were measured to examine growth-stimulating substances for LB which were derived from KM.

Materials and Methods

1. Materials

Lactobacillus rhamnosus, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus paracasei* subsp. *tolerans* (LB) and *Lactobacillus curvatus*, the strains of lactic acid bacteria isolated from chigo, and *Kluyveromyces marxianus* var. *lactis* (KM), the strain of yeast from chigo, were used in this experiment. Lactic acid bacteria and yeast were stored using GAM agar medium (Nissui Co.) and YM agar medium (Eiken Co.), respectively, for subsequent experiments.

2. Combination of strains

Using skim milk sterilized at 110°C for 5 minutes, a 10% reconstituted skim milk medium (10% RSM medium) was prepared. One of the strains of lactic acid bacteria and KM were inoculated to the medium to create an initial cell density of 10^6 /ml and 10^5 /ml, respectively and cultured together to determine the most efficient combination of bacteria and yeast in terms of lactic acid production. In general, microorganisms were cultured at 32°C.

3. Determination of viable cell count, lactic acid production and ethanol production

The selected strains of lactic acid bacteria and yeast were cultured individually or combined. The number of viable cells of lactic acid bacteria was measured on a plate-count agar with BCP (Nissui Co.), and the number of viable yeast cells was measured on potato dextrose agar (Eiken Co.). Lactic acid bacteria and yeast were incubated for 72 hours at 32°C and at 28°C, respectively. The culture was diluted adequately and 4 plates were prepared for each diluted culture. The number of viable cells was determined from the mean number of colonies observed on the 4 plates. Lactic acid production was measured by HPLC (Shimazu 6A) equipped with the Waters column (organic acid column, 7.8 mm × 30 cm). Ethanol production was measured by GC (Hitachi G 3000) equipped with a PEG 6000-support column.

4. Nutrient requirements of lactic acid bacteria

We conducted omission tests using Tamura-Kakuta medium¹⁶⁾ to investigate the amino acids and vitamins requirements of lactic acid bacteria. L-Amino acids at a concentration of 0.01% and vitamins were added to 10% RSM medium individually or combined, and LB was inoculated to the medium to create an initial cell density of 10^6 /ml. Nutrient requirements were examined on the basis of lactic acid production. The amino acids used in this experiment were those considered to be responsible for proliferation of LB, and the concentration of vitamins added to the medium was determined based on the report of Morishita *et al.*¹⁴⁾.

5. Growth factors of lactic acid bacteria

In order to investigate the effect of yeast on growth of LB and lactic acid production in the mixed culture,

one of culture media of yeast, cell-free extract of yeast was prepared by the method of Higashio *et al.*³⁾. After culturing yeast in a YM medium, the supernatant portion obtained by centrifugation was used as the culture medium of yeast. For preparation of a cell-free extract of yeast, the yeast bacteria that precipitated during centrifugation were collected and ground in a mortar added with distilled water, and the solution was filtered through a 0.2 μm membrane filter and their heated (sterilized at 110°C for 5 minutes) products was solublized in 10% RSM medium containing 30% of the total volume of the mixed medium, consisted of each solutions. To the medium prepared as described above, LB was inoculated to create an initial cell density of $10^6/\text{ml}$, and the amount of lactic acid produced in the culture was measured by an automatic acidity titrator (Mitsubishi GT-05).

6. Amino acid analysis by HPLC

Lactic acid bacteria and yeast were inoculated to 10% RSM medium to create an initial cell density of $10^6/\text{ml}$ and $10^5/\text{ml}$, respectively, and cultured either individually or combined. To measure the amount of free amino acids, 1.5 volume of 5% TCA was added to 500 μl of culture broth and mixed thoroughly. After centrifugation at 12,000 rpm for 2 minutes, 650 μl of the supernatant was taken and mixed with an equal volume of lithium citrate buffer (pH 2.2). The mixture was filtered through a 0.45 μm membrane filter, and the filtrate was used as the sample for amino acid analysis.

For peptide analysis, 2 ml each of culture broth was

mixed with 1 ml of 5% TCA, and the mixture was kept at 4°C for 12 hours. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was filtered through a 0.2 μm membrane filter. To 0.4 ml of the filtrate placed into an ampule, HCl was added to obtain a final concentration of 6 N HCl. After the ampule was sealed under reduced pressure by a vacuum pump, the contents were hydrolyzed at 110°C for 22 hours. After cooling, 1 ml of 6 N HCl was put into the ampule, and the solution was evaporated to dryness at 45°C using an aspirator. This ampule was then kept in a desiccator containing NaOH for 12 hours to remove completely the HCl. Then 650 μl of the supernatant was taken and mixed with an equal volume of lithium citrate buffer (pH 2.2), and the solution was filtered through a 0.45 μm membrane filter. The filtrate was used as the sample for amino acid analysis. Therefore, this sample also contains free amino acids. The true quantity of amino acid derived from peptides was calculated by subtracting the quantity of free amino acid from the measured value.

Analysis of amino acids was performed by HPLC-amino acid analyzing system (Shimazu 10 A) equipped with a column designed for amino acid measurement (Shim-pack/Amino-Li) with a guard column (Shim-pack/ISC 30/S0 50 4 Li) as an ammonia trap.

Results and Discussion

Lactic acid production by the strains of lactic acid bacteria and yeast isolated from chigo was measured

Table 1. Lactic acid production by single of *Lactobacillus* spp. and mixed culture with *K. marxianus* var. *lactis*

Species	Lactic acid (%)	
	Single culture	Mixed culture
<i>Lactobacillus rhamnosus</i>	0.43	0.70
<i>Lactobacillus paracasei</i> subsp. <i>tolerans</i>	0.49	1.07
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	0.48	0.85
<i>Lactobacillus curvatus</i>	0.29	0.76
<i>Kluyveromyces marxianus</i> var. <i>lactis</i>	0.10	—

One of the strains of lactic acid bacteria and *K. marxianus* var. *lactis* were inoculated to the 10% RSM medium to create an initial cell density of $10^6/\text{ml}$ and $10^5/\text{ml}$.

Incubation was performed at 32°C for 48 hrs.

Fig. 1. Changes of viable cell number, lactic acid and ethanol in single and mixed culture by *L. paracasei* subsp. *tolerans* and *K. marxianus* var. *lactis*. Using skim milk medium, LB and KM were inoculated to the 10% RSM medium to create an initial cell density of 10^6 /ml and 10^5 /ml.

Viable cell number by LB (□), and in mixed culture with KM (■)

Viable cell number by KM (○), and in mixed culture with LB (●)

Lactic acid production by LB (△), and in mixed culture with KM (▲)

Ethanol production by KM (◇), and in mixed culture with LB (◆)

in the single and mixed cultures. Results indicated that the lactic acid production was higher in the mixed culture of any combinations including LB than in the single culture of each strain of lactic acid bacteria.

Table 2. Stimulating effect of amino acids on lactic acid production by *L. paracasei* subsp. *tolerans*

Amino acids	Lactic acid (%)
Mixed amino acids*	0.80
Alanine	0.32
Valine	0.35
Phenylalanine	0.40
Serine	0.60
Threonine	0.34
Glutamic acid	0.73
Lysine	0.38
Arginine	0.37
Control	0.30

* A total of 0.08 mg/100 ml of eight amino acids was added.

0.01 mg/100 ml of each amino acids was added.

Incubation was performed at 32°C for 48 hrs.

Table 3. Stimulating effect of vitamins on lactic acid production by *L. paracasei* subsp. *tolerans*

Vitamins	Lactic acid (%)
Mixed Vitamins*	0.48
Thiamin	0.25
Riboflavin	0.27
Niacin	0.40
Pantothenic acid	0.27
Biotin	0.26
Pyridoxine	0.22
Folic acid	0.40
Control	0.26

* Seven vitamins were added in mixed vitamins.

Thiamin 0.1 mg, riboflavin 0.1 mg, niacin 0.1 mg, pantothenic acid 0.1 mg, biotin 0.01 mg, pyridoxine 0.2 mg, folic acid 0.01 mg.

Incubation was performed at 32°C for 48 hrs.

Thus, it is revealed that lactic acid production is stimulated most significantly in the mixed culture containing LB from chigo. A part of the results are shown in Table 1. While the concentration of lactic acid produced in the single culture of LB was 0.49% after 48 hours of incubation, the concentration of lactic acid in the mixed culture of LB and KM was

Fig. 2. Effect of amino acids and vitamins on lactic acid production by *L. paracasei* subsp. *tolerans*. Eight amino acids added (■), seven vitamins (▲), eight amino acids and seven vitamins (●), control (○).

Fig. 3. Effect of culture medium of yeast and cell-free extract of yeast of *K. marxianus* var. *lactis* on lactic acid production by *L. paracasei* subsp. *tolerans*.

Culture contents 30% each of culture medium of yeast and cell-free extract of yeast

Culture medium of yeast added (□), heated culture medium of yeast (■), cell-free extract of yeast (△), heated cell-free extract of yeast (▲), control (○).

1.07%, the highest value among the combinations.

The number of viable cells, lactic acid production and ethanol production were measured in the single and mixed cultures of LB and KM. The results are shown in Fig. 1. After 2 days of incubation, the number of viable LB cells in the mixed culture was 10-fold higher than that in the single culture. After 3 days of incubation, the number of viable cells reached a maximum and after 5 days or more decreased in both mixed and single cultures.

The number of viable KM cells in the single culture was higher at any time during incubation than that in the mixed culture. While the concentration of lactic acid was 0.70% after 3 days of incubation in the single culture LB, the concentration of lactic acid was as high as 1.82% in the mixed culture, but no significant difference in lactic acid production was observed between the single and mixed cultures after 7 days or more. Ethanol production was higher in the single KM culture than in the mixed culture at all times during incubation; for example, it was 1.87% in the single culture and 1.28% in the mixed culture after 3 days of incubation. These results indicated that co-existence of KM and LB affects the number of viable LB cells and lactic acid production, but not the number of viable KM cells and ethanol production. Furthermore, increases in the number of viable LB

cells and lactic acid production became less significant in the mixed culture as the initial KM : LB ratio increased (data not shown). When the incubation temperature was changed to 25°C or 28°C, the number of viable LB cells and lactic acid production was higher in the mixed culture than in the single culture, but the extent of the increase was reduced as the temperature decreased.

It is a well known fact that *lactobacilli* are highly auxotrophic and therefore require various nutrients for their growth. We conducted omission tests using abundance inside the yeast bacteria and examined the amino acid and vitamin requirements of LB using the Tamura-Kakuta medium¹⁶⁾. As a result, LB required eight L-amino acids : Ala, Val, Phe, Ser, Thr, Glu, Lys and Arg. This pattern of amino acid requirement is consistent with that reported by Guirard²⁾ and Morishita *et al.*¹³⁾. Seven vitamins : thiamin, riboflavin, niacin, pantothenic acid, biotin, pyridoxine and folic acid were required for LB.

The effect of each amino acid on lactic acid production on LB was examined when each of the eight

Table 4. Free amino acid concentraions in single and mixed

Amino acid	RSM medium	24 h			48 h	
		<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>
Asp	—	0.31	—	0.22	—	0.07
Thr	0.10	0.29	—	0.18	0.54	0.56
Ser	0.10	0.14	0.10	0.08	0.77	0.19
Glu	4.30	5.01	2.37	5.50	6.03	0.75
Pro	0.20	1.34	0.54	2.70	3.15	1.92
Gly	0.70	0.67	0.34	0.53	0.94	1.04
Ala	0.30	1.42	—	2.28	0.41	1.16
Val	0.10	0.28	—	0.38	0.90	1.50
Ile	—	0.11	—	0.18	0.39	1.07
Leu	—	0.24	—	0.76	1.01	0.14
Tyr	0.80	1.09	0.73	1.23	2.15	0.73
P-ala	—	0.12	—	0.22	0.50	—
Lys	0.20	1.24	—	0.90	3.09	—
Arg	—	0.58	—	1.10	2.00	—
Total	6.80	12.84	4.08	16.26	21.88	9.13

Table 5. Amino acid in acid hydrolyzate of single and mixed culture of

Amino acid	RSM medium	24 h			48 h	
		<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>
Asp	3.23	3.83	3.82	3.40	4.88	5.40
Thr	3.81	4.73	4.20	4.39	5.40	6.18
Ser	5.31	6.13	5.66	5.22	6.52	9.95
Glu	4.30	20.49	17.89	17.95	26.62	29.11
Pro	5.56	7.65	7.05	6.85	9.18	11.09
Gly	3.13	3.83	3.76	3.20	3.35	5.07
Ala	2.50	3.81	3.10	3.42	7.74	4.56
Val	3.01	4.55	3.83	3.96	5.44	7.17
Ile	—	4.30	3.92	3.81	4.81	7.29
Leu	3.28	4.02	3.63	3.13	5.23	6.70
Tyr	—	—	—	—	—	0.31
P-ala	17.51	2.19	2.19	2.04	1.91	3.01
Lys	4.39	5.87	4.75	4.45	5.99	10.05
Arg	2.68	4.68	14.43	2.86	3.21	4.63
Total	58.71	76.08	78.23	64.68	90.28	110.52

Microbial Relationship in Chigo, Inner Mongolia

culture with *L. paracasei* subsp. *tolerans* and *K. marxianus* var. *lactis*

72 h			96 h			
Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix
mg/100 ml						
0.04	1.21	0.18	0.30	2.70	0.40	0.29
2.72	1.06	0.55	3.64	3.69	1.30	3.57
2.64	0.72	0.19	1.68	2.00	0.60	1.70
4.69	9.90	0.81	1.33	9.73	1.00	1.59
16.44	6.19	1.14	19.21	16.74	3.60	18.80
2.46	0.75	2.10	5.15	0.86	2.70	5.18
23.70	6.19	1.95	35.48	12.70	7.20	36.38
2.56	2.53	0.65	2.43	4.57	0.60	2.44
0.97	1.03	0.54	—	2.24	0.30	0.69
2.82	3.37	—	—	6.52	0.20	1.05
1.54	2.96	0.70	0.26	2.33	0.60	0.96
2.46	1.17	—	—	1.95	0.10	0.33
1.27	4.78	—	—	5.80	—	—
—	2.62	—	—	2.84	—	—
64.31	44.48	8.81	69.48	74.67	18.60	72.98

L. paracasei subsp. *tolerans* and *K. marxianus* var. *lactis*

72 h			96 h			
Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix	<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>K. marxianus</i> var. <i>lactis</i>	Mix
mg/100 ml						
5.63	6.69	7.16	6.10	7.67	7.02	5.71
6.43	8.77	7.15	8.96	10.15	9.68	8.23
7.55	9.81	10.47	10.28	10.60	14.29	9.50
27.02	41.04	31.78	29.42	53.78	42.21	27.94
10.87	15.48	11.24	15.51	15.93	13.44	13.17
3.72	4.54	4.68	5.47	3.83	4.99	4.68
—	—	4.58	27.88	—	2.35	22.82
6.32	9.56	7.12	10.42	9.75	9.76	6.99
6.02	7.62	7.14	8.11	8.53	10.26	6.25
4.71	7.92	7.85	6.04	6.56	11.39	6.53
—	0.18	1.06	0.26	—	1.57	1.33
3.89	1.58	4.39	4.76	4.48	6.04	3.95
8.27	10.09	10.27	10.28	11.59	13.82	10.50
4.33	5.10	4.86	5.14	4.98	6.25	4.96
94.76	128.38	119.75	148.63	147.85	153.07	132.56

amino acids described above was added to a 10% RSM medium. As shown in Table 2, the concentration of lactic acid produced was 0.60% and 0.73% after two days of incubation in the presence of Ser and Glu, respectively, suggesting that Ser and Glu are the most important amino acids for proliferation of LB.

Since vitamins are also present in skim milk, we added only vitamins to RSM medium (0.1 mg/100 ml), based on the report of Morishita *et al.*¹⁴⁾ The results are shown in Table 3. The concentration of lactic acid after 48 hours of incubation was 0.40% for both niacin and folic acid when added to the medium. Although, lactic acid bacteria generally require niacin and folic acid as essential vitamins Koser⁷⁾, this experiment revealed that while LB used in this study required both niacin and folic acid for its growth, there was a higher requirement for folic acid composed with that reported by Koser⁷⁾. In addition, when LB was incubated at a lower temperature, *i.e.*, at 25°C or at 28°C, the pattern of nutrient requirements was essentially identical to that at 32°C. Therefore, the nutrient requirements of LB do not seem to be influenced by the incubation temperature.

Effects of the combinations of eight amino acids and seven vitamins on lactic acid production are shown in Fig. 2. When all the amino acids and vitamins were added in singles or mixtures to the 10% RSM medium, a significant increase in lactic acid production was observed except for thiamin and pyridoxine. That is, the concentration of lactic acid was 0.82% in the presence of all the amino acids and vitamins and 0.35% when none of the amino acids and vitamins was added. This result suggests an association of these amino acids and vitamins with LB proliferation.

To investigate the influence of KM on the number of viable cells of LB and lactic acid production in the mixed culture, one of culture media of yeast, cell-free extract of yeast, which were prepared by the method of Higashio *et al.*³⁾, and their heated products were added to 10% RSM medium, and the amount of lactic acid produced in the mixed medium was measured. As shown in Fig. 3, while the concentration of lactic acid produced was 0.70% after 72 hours of incubation in the medium without any solutions prepared from the yeast-culture broth, the concentration of lactic

acid was 1.42% and 1.63% in the presence of culture medium of yeast, cell-free extract of yeast, respectively, and 1.35% in the presence of any of the heated solutions. It was thought that substances that contribute to the growth of lactic acid bacteria exist in abundance inside the yeast bacteria.

In all the cases, lactic acid production increased, although the amount of lactic acid produced in the medium containing the heated solution was slightly lower compared with those for the media containing unheated solutions. These results suggest that water-soluble, relatively heat-stable substances derived from culture medium of yeast, cell-free extract of yeast may be responsible for the increased production of lactic acid in the mixed culture.

We measured the quantity of amino acid derived from peptides in both cultures of lactic acid bacteria alone and in mixed cultures of lactic acid bacteria and yeast. As shown in Table 4, After 48 hours of incubation, the amount of free amino acids was 9.1 mg/100 ml in the single KM culture, 21.9 mg/100 ml in the single LB culture, and 64.3 mg/100 ml in the mixed culture. The significant increase of free amino acids in the mixed culture was thought to be associated with the high activity of proteases detected in LB. In the mixed culture, the production of Pro and Ala was stimulated most significantly : 16.4 mg/100 ml for Pro and 23.7 mg/100 ml for Ala. However, the amount of these free amino acids were considered to be insufficient for a rapid proliferation of LB during the early phase of growth in the mixed culture.

To elucidate the relationship between the amount of nutrient and proliferation of lactic acid bacteria, the absolute quantity of amino acid derived from peptides and proteins was calculated by subtracting the quantity of free amino acid from the measured value, since free amino acid is included in these measurements. As shown in Table 5, the amounts of peptides were high in both single and mixed cultures after 48 hours of incubation : 110.5 mg/100 ml in the single KM culture, 90.3 mg/100 ml in the single LB culture, and 94.8 mg/100 ml in the mixed culture and remained at high levels thereafter. These results suggest that the large amount of peptides in a mixed culture may be responsible for the higher number of viable cells and

increased production of lactic acid in a mixed culture containing *L. paracasei* subsp. *tolerans* and *K. marxianus* var. *lactis*, which were isolated from chigo, than in a single LB culture. Further study of vitamins and other trace nutrients may be thought to contribute for the growth of lactic acid bacteria, is being planned.

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