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Isolation of lactic acid bacteria from seaweed and seagrass in the Japanese coastal waters and possible application of their soy milk fermentation products

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Abstract — Lactic acid bacteria were isolated from seaweed and seagrass samples collected from various sea areas around Japan. Out of 208 isolates, 24 strains were catalase negative and showed clear holes on the agar media containing calcium carbonate due to the production of lactic acid, suggesting that these strains were considered to be lactic acid bacteria. Among these, 3 strains showed high production of lactic acid and were selected for further studies. These strains were found to be *Lactobacillus delbrueckii* subsp. *lactis* based on the 16S rDNA sequence analysis. The species is well-known as a plant-derived lactic acid bacterium of terrestrial origin. Physiological and biochemical characteristics of the three strains and their type strain (NBRC3202) were compared. Growth temperature and utilization of amygdalin, arbutin and salicin of the three strains were different from their type strain. The supernatant of fermented commercial soy milk of three isolated strains showed acceleration of synthesis of collagen and hyaluronic acid on normal human skin fibroblast. Among these strains, strain KM-2, which was isolated from Kumejima Town, in Okinawa Prefecture showed highest acceleration activities.

Key words: Lactic acid bacteria, *Lactobacillus delbrueckii*, DGGE, soy milk, hyaluronic acid, collagen

Introduction

Lactic acid bacteria are beneficial microorganisms for humans, aiding in the maintenance of intestinal homeostasis (Saez-Lara et al. 2015). They have been isolated from various sources such as terrestrial soil and animal bodies and have been utilized for various purposes including fermented foods. The primary industrial uses of lactic acid bacteria include fermented foods like yogurt (Kobayashi et al. 2024) and pickles (Banik 2023). In recent years, live lactic acid bacteria have gained attention as probiotics for reducing the risk of various diseases (Das et al. 2022). They are also incorporated into cosmetic products such as fermented soy milk and whey for hair care (Ichioka -Mori et al. 2022).

However, lactic acid bacteria used in cosmetics have been terrestrial in origin, with no use of marine-derived strains. This is due to challenges in sampling marine environments, leading to a lag in research compared to terrestrial microorganisms. The marine environment covers about 70% of the Earth's surface and exhibits distinct characteristics like high salt content, high hydrostatic pressure, low temperature, and low organic matter concentration, making it a rich source of diverse microorganisms and unique metabolites (Imada, 2004; Imhoff et al. 2011). In these two decades, various marine microorganisms which produce valuable natural products have been successively isolated from oceans worldwide, leading to an increasing spotlight on marine microorganisms (Jensen et al. 2005).

Frantzmann et al. (1991) first isolated lactic acid bacteria from marine environments. Subsequently, Ishikawa et al. (2003) isolated a novel species of lactic acid bacteria with salt and alkaline tolerance, naming it "*Marinilactibacillus psychrotolerans*." Since then, research institutions have been exploring marine lactic acid bacteria, elucidating their ecology and taxonomic characteristics. However, there have been no studies on the practical application of marine lactic acid bacteria in various industries. This study not only investigated the physiology of marine lactic acid bacteria but also focused on their isolation.

Under these circumstances, the present study devised a method to isolate marine lactic acid bacteria. Enrichment cultures were conducted on various marine samples, followed by extraction of microbial DNA from the resulting cultures. Lactic acid bacteria were detected using denaturing gradient gel electrophoresis (DGGE). Subsequently, lactic acid bacteria were isolated and cultured through cultivation methods from samples where multiple types of lactic acid bacteria were detected. Promising strains exhibiting high lactic acid production capability were selected from the obtained lactic acid bacterial isolates, and their taxonomic char-

acteristics were elucidated. Furthermore, soy milk fermentation broth was prepared using these promising strains. The resulting fermentation broth was then examined for its effects on cell viability, hyaluronic acid, and collagen production using human dermal fibroblast cells.

Materials and Methods

Collection of seaweed and seagrass samples

The seaweed and seagrass samples collected from the coastal areas of Japan were utilized as sources for isolating marine lactic acid bacteria. The sampling areas and collection dates were as follows: Otsuchi Town, Iwate Prefecture in June 2009 and August 2010; Shimizu Ward, Shizuoka City in March 2009; Minamiboso City, Chiba Prefecture from September 2008 to August 2014; and Kumejima Town, Okinawa Prefecture on November 17, 2010. A total of 24 samples, comprising green algae (11), brown algae (6), red algae (1), and seagrass samples (6) from various locations in the coastal areas of these points were obtained.

Enrichment culture and extraction of bacterial DNA from seaweed and seagrass sample

Approximately 1 g (wet weight) of seaweed and seagrass samples collected from various marine areas were suspended in 9 ml of sterile seawater. The suspension was appropriately diluted and subjected to three-day enrichment culture at 27°C in MRS liquid medium (Kanto Chemical) prepared in freshwater or seawater and GYP liquid medium (Difco) prepared in seawater. To this, 600 μL of TE buffer and 20 μL of lysozyme (Wako, 5 mg mL^{-1}) were added and kept at 37°C for 1 h. Then, 3 μL of Proteinase K solution (Invitrogen, 20 μL mL^{-1}) and 30 μL of 10% sodium dodecyl sulfate were added respectively, followed by incubation at 37°C for 1 h. A mixture of phenol–chloroform–isoamyl alcohol (25 : 24 : 1) was added (600 μL), and the bacterial cells were disrupted using a bead homogenizer (MS-100R, Tomy Co.) at 2,000 rpm for 25 s. After homogenization, 100 μL of cetyl trimethylammonium bromide solution was added, thoroughly mixed, and incubated at 65°C for 10 min. The mixture was then centrifuged (20,000×g, 20 min), and the supernatant was collected. Isopropanol (600 μL) was added to the supernatant to precipitate DNA, which was then purified using a method described previously (Yang et al. 2019) and dissolved in 20 μL of ultrapure water before storing at −20°C.

Analysis of lactic acid bacteria by PCR-denaturing gradient gel electrophoresis (DGGE)

After thawing the DNA samples, 0.5 μL of each sample was taken and mixed with 12.5 μL of Go Taq (Promega), 10 μL of ultrapure water, and 1 μL each of the lactobacilli-specific primers (Lopez et al. 2003) WLAB1 (TCCGGATTT ATTGGGCGTAAAGCGA, Forward) and WLAB2 (CGC CCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCC CTCGAATTAAACCACATGCTCCA, Reverse) (with GC clamps). The PCR reaction was carried out by initially heating at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 30 min to obtain PCR products.

DGGE was performed by the method described previously (Yoshida et al. 2008). An 8% acrylamide gel solution was prepared by adding 10% ammonium persulfate and *N*, *N*, *N*′, *N*′-Tetramethylethylenediamine to achieve final concentrations of 0.9% and 0.09%, respectively. The gel was polymerized, and a gel with a denaturing gradient of 20–55% (100% denaturant consisting of 7 M urea and 40% formamide) was prepared using the Gradient Delivery System (Bio-Rad Co.). DGGE was performed using the D Code System (Bio-Rad Co.) with electrophoresis conducted at 60°C for 18 h at 100 V. The gel was then photographed using the Molecular Imager FX (Bio-Rad Co.), and the migration results were examined. Subsequently, bands in each sample were detected using image detection software (Lane Multi Screener ver. 3.0, Atto Co.), and band analysis was performed using image analysis software (Quantity One ver. 4.3.0, Bio-Rad Co.) to confirm the presence of lactic acid bacteria.

Isolation and cultivation of lactic acid bacteria from seaweed and seagrass samples

Lactic acid bacteria were isolated from seaweed and seagrass samples where their presence was confirmed through DGGE analysis. Specifically, 2 g (wet weight) of the seaweed or seagrass sample was mixed with 20 mL of sterile seawater in a sterile test tube and vigorously vortexed. The resulting suspension was further diluted appropriately with sterile seawater. Then, 0.1 mL of this diluted suspension was inoculated onto MR agar and GYP agar (pH 6.3, Kanto Chemical) supplemented with 1% calcium carbonate. Subsequently, the plates were incubated at 27°C for 7 days.

After incubation, colonies appearing on the agar plates were examined for the dissolution of calcium carbonate (halo formation). Colonies showing clear halo formation were individually picked and streaked onto MRS agar. Following cultivation, a few drops of 3% hydrogen peroxide were added to the colonies, and if no foaming was observed after a short period, they were identified as catalase-negative, indicating lactic acid bacteria. These selected strains were then collected as lactic acid bacterial strains and utilized for the following experiments.

Identification and physiological/biochemical characterization of high fermentation activity strains in soy milk

Among the obtained lactic acid bacterial strains, three strains exhibiting particularly high fermentation activity in soy milk were selected for species identification based on the 16S rDNA sequence. After anaerobic cultivation for 3 days at 37°C in MRS agar (in anaeropack), colonies were inoculated into MRS broth and cultured for 1 day at 37°C. Following cultivation, bacterial cells were collected by centrifugation (4°C, 20,000×*g*, 20 min), and the DNA was purified using the Soil DNA Isolation Kit, PowerSoil (Funakoshi). Subsequently, sequence analysis was conducted by outsourcing to TechnoSuruga Co., Ltd. The primers 9F and 1510R were used for PCR. The nucleotide sequences were analyzed using the ABI PRISM 3130 xl Genetic Analyzer System (Applied Biosystems), and sequence determination was carried out with ChromasPro 2 (Technelysium, AUS). For BLAST homology searches, the analysis software ENKI (TechnoSuruga Laboratory, Japan) was used. Additionally, the databases DB-BA 15.0 (TechnoSuruga Laboratory) and the international nucleotide sequence databases (DDBJ/ENA (EMBL)/GenBank) were utilized.

Additionally, the strain *Lactobacillus delbrueckii* subsp. *delbrueckii* NBRC3202 was purchased from the National Institute of Technology and Evaluation (Independent Administrative Institution), and physiological/biochemical characteristics were compared between these three selected strains and NBRC3202 strain after 24 and 48 h of cultivation at 37°C using a bacterial identification test kit (API50 CHL, BioMerieux, Marcy l'Etoile). The strain was the type strain of *Lactobacillus delbrueckii* subsp. *delbrueckii* until 2022.

Preparation of soy milk fermentation liquid

Commercially available organic soy milk (Marusan Co., Ltd.) without any adjustments in composition was obtained. Aseptically, 200 mL of the soy milk was transferred into sterile glass culture bottles with screw caps. Each bacterial strain was inoculated into the soy milk to achieve a final concentration of 4×10^5 cells mL^{-1}, and the bottles were then incubated at 37°C for 48 h. After incubation, the fermented soy milk was subjected to low-temperature sterilization at 60°C for 30 min, followed by centrifugation (16,770×*g*, 10 min, 4°C) to separate the supernatant, which was referred to as "soy milk fermentation liquid" in this study. The quantification of lactic acid produced in the soy milk fermentation liquid was carried out using a lactic acid assay kit (BIO Vision, USA), and the amount of lactic acid produced was calculated based on the standard curve of *L*-lactic acid.

Cytotoxicity assay of soy milk fermentation liquid

The cytotoxicity assay of soy milk fermentation liquid was evaluated using normal human dermal fibroblast cells derived from normal human skin (NB1RGB cells, passage number 12, obtained from the RIKEN BioResource Center). The assay utilized the enzymatic activity of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced to purple formazan dye.

For the assay, MTT was added to the cells, and the reduction of MTT to formazan dye was measured spectrophotometrically (Yamada et al. 2007). A blank control was performed using 0.5% FBS-containing Eagle's Minimum Essential Medium (MEM), while the negative control involved adjusting organic soy milk to pH 3.4 by adding lactic acid and then centrifuging to remove any solid particles, followed by using the obtained supernatant. Measurement was performed six times, and average values with standard deviations (SD) were used as the data.

Stimulation of collagen (CO) synthesis by soy milk fermentation liquid (Lopez and Rojkind, 1985)

NB1RGB cells (passage number 12) were seeded at a density of 20,000 cells per well in a 96-well plate and pre-cultured for 24 h at 37°C (5% CO_2) in Eagle's Minimum Essential Medium (MEM) containing 10% FBS (Nissui Pharmaceutical). Subsequently, soy milk fermentation liquid was added to the medium at a final concentration of 5%, and the cells were further incubated for 48 h at 37°C (5% CO_2) in Eagle's MEM containing 0.5% FBS for evaluation.

To evaluate the stimulation of collagen synthesis by soy milk fermentation liquid, NB1RGB cells (passage number 13) were seeded at a density of 20,000 cells per well in a 96-well plate and pre-cultured for 24 h at 37°C (5% CO_2) in Eagle's Minimum Essential Medium (MEM) containing 10% FBS. Subsequently, soy milk fermentation liquid was added to the medium at final concentrations ranging from 1.25% to 5%, and the cells were further incubated for 48 h at 37°C (5% CO_2) in Eagle's MEM containing 0.5% FBS for evaluation. After incubation, the culture medium was removed, and the cells were washed twice with 100 μL of PBS (−) per well. Following removal of PBS (−), the cells were incubated in 50 μL of staining solution per well in the dark for 1 h. After removal of the staining solution, the cells were washed five times with 100 μL of PBS (−) per well, and then incubated with 100 μL of extraction solution per well at 37°C for 1 h. Subsequently, the absorbance at 490 nm was measured using a microplate reader to quantify the collagen stain absorbance in the soy milk fermentation liquid. The same blank and negative controls used in the aforementioned cytotoxicity assay were employed for this evaluation as well. Magnesium *L*-ascorbyl phosphate (APMg) was used as a positive control (Lopez and Rojkind, 1985).

Results

Detection of lactic acid bacteria from seaweed and seagrass samples by DGGE

Out of a total of 24 seaweed and seagrass samples tested, lactic acid bacteria bands were observed in 12 samples through DGGE analysis. Fig. 1 shows the results obtained from a sample of Eelgrass (*Zostera marina*) collected in Kumejima Town. As evident from this figure, lactic acid bacteria bands were observed in all three media. Therefore, it was decided to isolate lactic acid bacteria from these samples. As an example of the DGGE results, obtained from a sample of Eelgrass (*Zostera marina*) collected in Kumejima Town. The lactic acid bacteria bands were observed in all three media: MRS liquid medium (freshwater and seawater) and GYP liquid medium (seawater). However, the intensity and appearance pattern of the bands varied depending on the medium. In this study, it was decided to isolate lactic acid bacteria from the enrichment culture where the densest bands were observed, which, in this figure, corresponded to GYP and seawater.

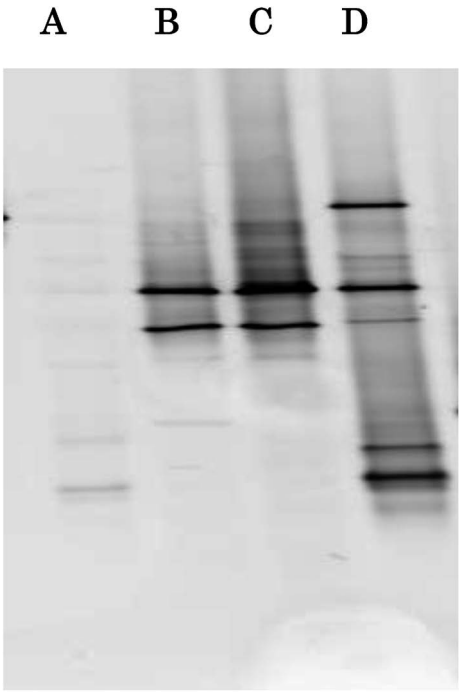

Fig. 1. DGGE results of eelgrass isolated from Kumejima Town. A; Before enrichment, enriched by B; MRS (Freshwater), C; MRS (Seawater), D; GYP (Seawater).

Isolation of lactic acid bacteria from seaweed and seagrass samples

A total of 208 bacterial strains showing calcium carbonate solubility were isolated from 10 mL of enrichment media, including MRS agar (freshwater and seawater) and GYP agar (seawater). Upon conducting catalase tests on these isolated strains, 24 strains tested negative, thus confirming them as lactic acid bacterial strains. Table 1 shows the number of lactic acid bacterial isolates and the isolation rate per each sample. As evident from this table, a significant number of lactic acid bacteria was isolated from green algae (11 strains, 18.0%), whereas fewer were isolated from brown algae (6 strains, 10.7%). Moreover, as illustrated in Table 2, while the highest number of lactic acid bacteria was isolated from Otsuchi Town (12 strains, 13.5%) whereas none were isolated from Shimizu award.

Table 1. The number of strains dissolved calcium carbonate and catalase negative strains from seaweeds and seagrass.

Seaweed or seagrass	The number of strains dissolved calcium carbonate (A)	The number of catalase negative strains (B)	B/A×100 (%)
Green algae	61	11	18.0
Brown algae	56	6	10.7
Red algae	39	1	2.6
Seagrass	52	6	11.5
Total	208	24	11.5

Table 2. The number of strains dissolved calcium carbonate and catalase negative strains from various sea areas.

Sea areas	The number of strains dissolved calcium carbonate (A)	The number of catalase negative strains (B)	B/A×100 (%)
Otsuchi Town	89	12	13.5
Minamiboso City	91	9	9.9
Shizuoka Shimizu Ward	10	0	0
Kumejima Town	18	3	16.7
Total	208	24	11.5

Table 3. Identification of lactic acid bacteria by 16S rDNA sequencing.

Strain No.	Collection location/ Collection date	Sample name	Number of base analyzed	Homology (%)
TI-13	Minamiboso City May 15, 2009	Aosa (*Ulva* sp.)	1,495	100
KM-1	Kumejima Town November 17, 2010	Eelgrass (*Zostera marina*)	1,495	99.87
KM-2	Kumejima Town November 17, 2010	Eelgrass (*Zostera marina*)	1,471	99.73

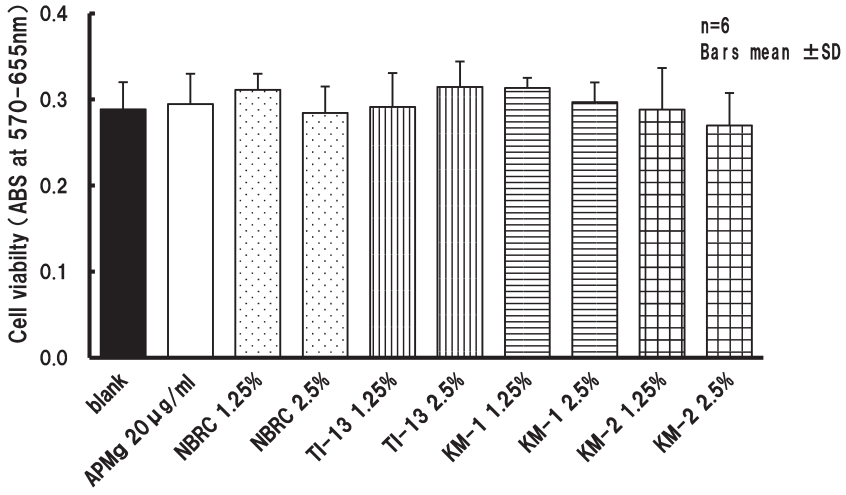

Fig. 2. The effect of fermented soy milk supernatant of various strains on cell viability (NB1RGB).

Taxonomic characteristics of highly active lactic acid bacteria

Three strains exhibiting high calcium carbonate dissolution activity among the 24 lactic acid bacterial strains were selected and designated as strain TI-13-1 (Minamiboso City), strain KM-1 (Kumejima Town), and strain KM-2 (Kumejima Town), respectively. Species identification based on the 16S rDNA sequence revealed that all three strains were closely related to *Lactobacillus delbrueckii* subsp. *delbrueckii*, showing 99.92%, 99.9%, and 99.86% sequence similarity for TI-13-1, KM-1, and KM-2 strains, respectively, as shown in Table 3. Subsequently, the standard strain *L. delbrueckii* subsp. *delbrueckii* NBRC3202 was obtained, and parallel experiments were conducted with these three strains. The results revealed differences between these three strains and the standard strain, such as positive growth at low temperatures (15°C) and negative utilization of amygdalin, arbutin, and salicin (data are not shown). Furthermore, all three strains exhibited efficient fermentation of soy milk, resulting in a pH range of 4.1–4.2 in the soy milk fermentation liquid and the production of 103–128 mM of lactic acid (data are not shown).

Cell viability of soy milk fermentation liquid

Fig. 2 illustrates the cell viability of soy milk fermentation liquid. As evident from this figure, no cytotoxicity was observed towards NB1RGB cells for any of the lactic acid bacteria tested. Additionally, there was no observed activation of cell activity.

Promotion effect of soy milk fermentation liquid on collagen synthesis

The promotion effect of soy milk fermentation liquid on collagen synthesis is depicted in Fig. 3. As evident from the figure, no promotion effect was observed in the unfermented soy milk. However, all marine-derived lactic acid bacteria exhibited higher collagen synthesis promotion effects compared to their respective standard strains. Particularly, strains isolated from marine sources showed higher promotion effects than the standard strains, with the 1.25% concentration showing a higher promotion effect than the 2.5% concentration. Among these strains, strain KM-2 isolated from Kumejima Town exhibited the highest promotion effect. The sequence of 16S rDNA of strains TI-13, KM-1 and KM-2 have been registered in the database with the accession Nos. of LC 192952, 192950, and LC192951, respectively.

Discussion

In this study, lactic acid bacteria were isolated from var-

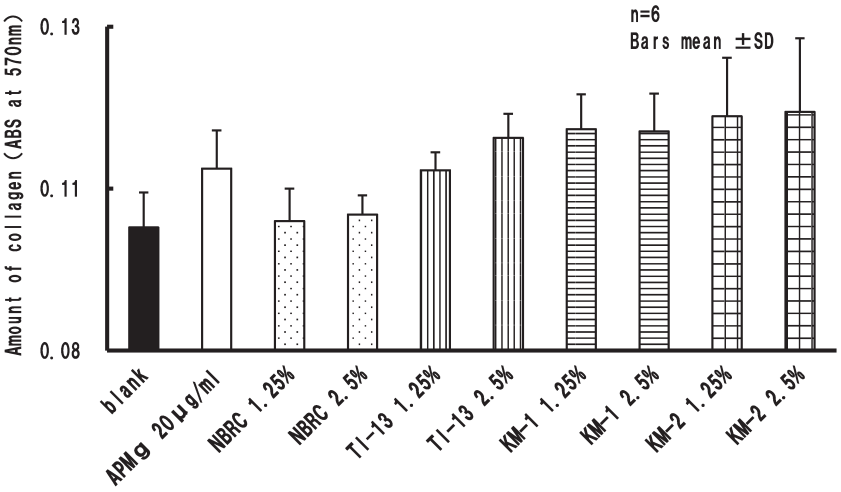

Fig. 3. The amount of collagen production in cultured fibroblast cells as influenced by sample addition.

ious marine areas by performing enrichment cultivation and then extracting microbial DNA to examine the presence of lactic acid bacteria bands using DGGE. Lactic acid bacteria were isolated from the enrichment medium where dense bands were predominantly observed. As a result, a total of 24 bacterial strains showing positive results in calcium carbonate dissolution and negative results in catalase tests were obtained and classified as lactic acid bacteria strains. Among these 24 strains, we selected three strains (TI-13-1 strain isolated from Minamiboso City, KM-1 strain from Kumejima Town, and KM-2 strain also from Kumejima Town) with particularly high calcium carbonate dissolution activity for further experiments. Species identification based on the 16S rDNA sequence revealed that all these strains were closely related to *Lactobacillus delbrueckii* subsp. *delbrueckii*. However, these strains exhibited differences from standard strains in terms of positive growth at low temperatures (15°C) and negative utilization of amygdalin, arbutin, and salicin.

L. delbrueckii is a well-known lactic acid bacterium that plays a significant role in the fermentation of various animal and plant-based food products, and currently, four subspecies have been reported (Kudo et al. 2012). *L. delbrueckii* subsp. *bulgaricus* is mainly isolated from yogurt (Michaylova et al. 2007), *L. delbrueckii* subsp. *delbrueckii* is found in fermented plants (Germond et al. 2003), *L. delbrueckii* subsp. *indicus* is isolated from fermented milk in India (Dellaglio et al. 2005), and *L. delbrueckii* subsp. *lactics* is isolated from cheese (Weiss et al. 1983).

The cell viability and promotion effects on the production of important matrix components in the cosmetic field, such as collagen (CO protein) and hyaluronic acid (HA), by soy milk fermentation broth of these three strains derived from the ocean were examined using human dermal fibroblasts. While unfermented soy milk showed no promotion effect, both marine-derived lactic acid bacteria and standard strains exhibited promotion effects. Notably, strain T1–13 showed the highest effectiveness. Moreover, as collagen decreases with age in the dermis, resulting in loss of skin elasticity and increased wrinkles, increasing collagen production in the dermis could help maintain firm and wrinkle-free skin (Varani et al. 2006). Since the marine-derived lactic acid bacteria strains exhibited higher promotion effects on collagen production compared to their land-derived counterparts, it is suggested that they could be utilized in cosmetics. Soy milk contains a large amount of glycoside type isoflavones such as genistin and daidzin). Fermentation can convert these compounds into aglycone type isoflavones (genistein and daidzein) with higher bioactivity. Aglycone type isoflavones bind to estrogen receptors and exert estrogen-like effects. Estrogen is known to maintain skin elasticity and promote collagen synthesis (Izumi et al. 2007).

In conclusion, the author isolated marine lactic acid bacteria through enrichment cultivation and DGGE methods. The fermented soy milk produced by these isolated strains showed higher abilities in promoting hyaluronic acid and collagen production than terrestrial lactic acid bacteria strains. This demonstrates the diverse physiological functions of marine lactic acid bacteria. Cosmetic ingredients using lactic acid bacteria have already been registered with over 100 names (Chiba 2007) and it is expected that cosmetics containing these ingredients will continue to be widely distributed in the market. While marine-derived lactic acid bacteria as cosmetic ingredients are still relatively unknown, and many of their effects remain undiscovered, it is anticipated that they will be used in various ways as new cosmetic ingredients in the future.

These findings suggest the potential of marine lactic acid bacteria as novel cosmetic ingredients. Further investigations into their physiological and functional properties are warranted.

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