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Original

General properties of lactic acid bacteria from deep seawater and α-glucosidase inhibitory activity of fermented soymilk

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Abstract

Deep seawater (DSW) contains various resources useful for human life, including cold energy. In this research, we focused on lactic acid bacteria, which are expected to prevent diabetes, hypertension, and obesity. Thirty-two strains of lactic acid bacteria were isolated from Izu-Akazawa DSW. For comparison, 34 strains from surface seawater (SSW) or seaweed from other sources and 25 strains from commercial fermented food (FF) were prepared. All isolates from the three isolation sources (DSW, SSW, FF) were tested for cell growth potential by culture conditions and showed little difference in general characteristics. Next, considering the use of the isolate, the ability of the isolate to ferment soymilk was studied. As a result, there was a difference in the fermentability of soymilk among the isolates from each source. To study the function of soymilk fermented by the isolates, their inhibitory effect against α-glucosidase activity was evaluated. α-Glucosidase activity was well known as a key effector of diabetes, a national disease in Japan today. As a result, there were differences in the inhibitory effect on α-glucosidase activity of soymilk fermented by the isolates. The inhibitory effect of soymilk fermented by isolates from DSW on α-glucosidase activity was shown to be 5.0±0.7 units/mL, which was the strongest among the isolates from three kinds of sources ($p<0.05$, SSW; 4.2±1.7 units/mL, FF; 3.3±1.5 units/mL). Then, we performed 16S rDNA analysis of each isolate and identified it as a *Lactobacillus* species level. As a result, the lactic acid bacteria species isolated from each source had a characteristic, which was the difference in the content of *Lactiplantibacillus plantarum*. The content of *L. plantarum* in isolates was independent of soymilk fermentability but associated with inhibitory effect on α-glucosidase activity of fermented soymilk. This study suggested that DSW is a useful source for the isolation of certain lactic acid bacteria, especially *L. plantarum*. In the future, it is expected that the use of *L. plantarum* isolated from DSW will expand to the field of human health.

Key Words: α-glucosidase, diabetes, isolation source, lactic acid bacteria, *Lactiplantibacillus plantarum*

1. Introduction

It has been a long time since the era of gluttony came to Japan. As a result, the number of diabetic patients is increasing day by day. Diabetes is a serious disease that causes three major complications such as neuropathy, numbness in hands, retinopathy and deterioration of renal function. It is well known that the number of diabetic patients in developed countries is increasing day by day. Diabetes is classified into type 1 and type 2, and about 90% of people in Japan have type 2 diabetes. According to "The National Health and Nutrition Survey in Japan, 2016" (Ministry of Health, Labor and Welfare, 2016), there are about 10 million people who are strongly suspected of having diabetes (prevalent diabetes) and those who cannot rule out the possibility of diabetes

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(prediabetes group). There is concern that the number will continue to increase in the future, and the development of new therapeutic agents is required.

α-Glucosidase is an enzyme that decomposes disaccharides into monosaccharides and it is related to the blood glucose level. The inhibitors of the enzyme suppress the reaction, as a result it is possible to suppress the rapid rise in the blood glucose level and reduce the risk of diabetes (Hiraoka *et al.* 2016). Acarbose, which is well-known as the α-glucosidase inhibitor now, is a substance produced by some strains of actinomycetes such as *Streptomyces* and *Actinoplanes* (Kim *et al.* 2002; Goeke *et al.* 1996). Acarbose is used as an oral hypoglycemic agent to treat type 2 diabetes due to its α-glucosidase inhibitory activity. However, it has been reported that acarbose causes some side effects such as intestinal bloating and diarrhea after administration. For these reasons, new drugs on diabetes have been developed for the purpose of reducing side effects and improving drug efficacy, and also probiotic treatment methods by oral administration of live lactic acid bacteria is known. The inhibitory effect on α-glucosidase has been reported in various fermented products of lactic acid bacteria such as *Lactiplantibacillus plantarum*, *Lactobacillus fermentarum*, *Lacticaseibacillus casei and Lacticaseibacillus rhamnosus* strains isolated from various environments (Panwar *et al.* 2014).

Interestingly, it was reported that there was significant difference of the potency and spectrum of the inhibitory activity of *L. plantarum* between strains of a single species of *L. plantarum* (Panwar *et al.* 2014). Furthermore, some *Lactobacillus* extracts possessed a broader spectrum of activity than acarbose, also effectively inhibiting lactase, maltase, sucrase and amylase in addition to α-glucosidase (Panwar *et al.* 2014). These reports clearly demonstrate that oral administration of lactic acid bacteria can expect to reduce the blood glucose level *in vivo* (Panwar *et al.* 2014).

Until now, lactic acid bacteria have been isolated mainly from fermented foods such as kimchi and rice bran pickles, and feces. Recent studies have revealed that lactic acid bacteria living in neritic marine environment have different compositions, growth temperatures, and salt tolerances from the bacteria originated from fermented foods (Ishikawa *et al.* 2005; Suzuki *et al.* 2020).

However, there are very few reports on the isolation of lactic acid bacteria from deep seawater (DSW) at depths exceeding 200 m (Yamada *et al.* 2022). Since it is a harsh environment that cannot be seen on land, such as low temperature, high hydrostatic pressure and cleanliness (Fujita and Takahashi, 2006) , the existence of unique microorganisms is expected. It was known that the number of microorganisms in DSW was considerably smaller than that in surface seawater (Imada, 2012). In addition, management and operation of DSW is decentralized by prefecture. Real-life situations such as those described above add to the difficulty of microbial research in DSW. In the above situation, useful microorganisms from DSW are now expected to be used for various industries.

Therefore, in this study, we isolated lactic acid bacteria from DSW, which is rarely studied and examined the ability of fermentation on soymilk and evaluated α-glucosidase inhibitory activity of the fermentation broth. The aim of this study was to analyze the characteristics of lactic acid bacteria isolated from DSW in comparison with the bacteria isolated from other sources such as surface seawater environment (SSW) and fermented foods. As a result, some findings were obtained and are reported here.

2. Materials and methods

2.1. Lactic acid bacteria isolated from DSW

In this study, all of isolates from DSW were isolated from bag filter (BF) which removes suspended solid in DSW (mesh size 0.5 μm, diameter 180 mm, length 800 mm, R1045 type, Nagoya Filter Sales Co., Ltd.). BF was used as a suitable source of DSW-derived microbes (Yamada *et al.* 2022). At Izu-Akazawa, about 1,000 tons/month of DSW is taken from a depth of about 800 m

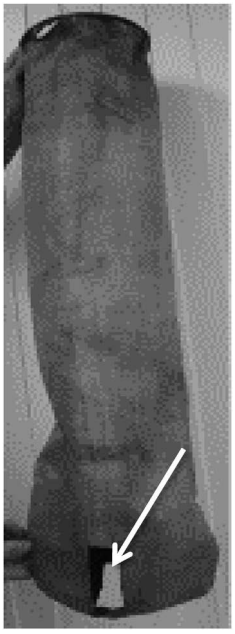

Fig. 1. Photograph of the bag filter (BF) fabricated for this study.
The size of BF is 180 mm in diameter and 800 mm in length. The arrows indicate two cutouts (width 30 mm, height 30 mm) at the bottom of the BF for collecting lactic acid bacteria.

and filtered by this BF. The BF used from January 2012 to February of the same year was used in this study. The bottom of this BF was aseptically cut into 30 mm × 30 mm squares and subjected to isolation of lactic acid bacteria (Fig. 1). For the comparison of sources from DSW, sources from surface sea water environment (SSW) such as seawater sampled directly above DSW, sea sand and seaweed from various regions including Kumejima Town, Okinawa Prefecture were applied. In addition, commercially available fermented foods such as kimchi and rice bran pickles (FF strain) were also applied as isolation sources for comparison. Each isolation source was smeared directly onto the following isolation medium. For the isolation of these lactic acid bacteria, MRS agar medium, which is widely used for lactic acid bacteria isolation, was used. Calcium carbonate (1%) was added to each strain. In addition, sample diluents were prepared individually for each isolation source, and half of the DSW and SSW corresponding to the isolates were mixed and used as the serial dilutions. After culturing, strains that were visually confirmed by the shape of the colony (convex shape, color, etc.) and the presence or absence of a transparent halo around the colony were picked as lactic acid bacteria candidate strains, and streak culture was performed. Three % hydrogen peroxide solution was added dropwise to the colonies of the obtained strains, and the strains that did not show foaming were judged to be lactic acid bacteria (Yamada *et al.* 2022). After thawing, the cells were pre-incubated (27℃, 1 day) on MRS agar medium and used for the experiment.

2.2. Preparation of soymilk fermented product of DSW-derived lactic acid bacteria and pretreatment for measurement of *α*-glucosidase inhibitory activity

Twenty mL of commercially available unadjusted soymilk (Marusan) was dispensed into each conical tube (50 mL /tube, Falcon, Corning International) and sterilized in an autoclave (121℃, 15 minutes). After sterilization, 200 μL of the DSW-derived lactic acid bacteria solution that had been pre-cultured for a day at 27℃ in MRS liquid medium was inoculated to these soymilk, and static culture was carried out at 37℃ for 3 days. For comparison, an artificially fermented soymilk product only adjusted at pH 4.1 ± 0.1 by adding lactic acid to unadjusted soymilk without inoculating lactic acid bacteria was prepared. It was used as a negative control in this experiment. Next, the inhibitory effect on α-glucosidase activity was evaluated by the following method. The supernatant of each fermented soymilk or artificially fermented soymilk was dispensed into each microtube (1.5 mL/tube) and centrifuged at 4℃, 20,630 × g for 10 minutes to prepare an evaluation sample.

An aliquot of 500 μL of each supernatant was taken and dried (50℃, 12–16 hours) using a heat block (Taiyo Kagaku Kogyo Co., Ltd.). An equal amount (500 μL) of 99.5% methanol (Fujifilm Wako pure chemical, special grade) was added and extracted. Those extracts were centrifuged again on the above condition. After collecting the supernatant, inhibitory activity against α-glucosidase was evaluated.

2.3. Alpha-glucosidase inhibitory activity of soymilk fermented by DSW-derived lactic acid bacteria

The α-glucosidase inhibitory activity of each supernatant from soymilk fermented by DSW-derived lactic acid bacteria was measured by the method of Watanabe *et al.* (1997) with slight modification. First of all, α-glucosidase solution (100 μL, 0.0015 mg/mL, Oriental Yeast Co., Ltd. 46510003) and 100 μL of the above samples for evaluation of α-glucosidase inhibitory activity were placed in a 96-well plate (Costar®). After dispensing, pre-incubation (37℃, 10 minutes) was performed. After adding 100 μL of *p*-Nitrophenyl-α-D-glucopyranoside (Fujifilm Wako pure chemical, 355–17471) to all of wells in the plate, the plate for evaluation was incubated at 37℃ for 30 minutes. After stopping the reaction, absorbance at 415 nm was measured using a microplate reader (Nippon Bio-Rad Laboratories, Model 550). α-Glucosidase inhibitory activity was calculated based on the ratio between the increased absorbance values indicated by a supernatant from fermented soymilk of a sample versus the increased absorbance value indicated by a supernatant of artificial fermented soymilk. Increasing of these values were caused by incubation for 30 minutes. The formula for calculating α-glucosidase inhibitory activity (IU/mL) is shown below.

One unit of the inhibitor (1 U) was defined as the amount of the inhibitor calculated according to the dilution ratio and the inhibitory activities of DSW-derived strains were compared with those of the SSW and FF strains, respectively. Acarbose (Fujifilm Wako pure chemical, 013–26971) was used for the positive control. Measurement was performed at $n = 3$, and the average value with standard deviation was used as the data.

$$\mathrm{IU} = \left(1 - \frac{\text{Increase of optical density at 415 nm of soymilk fermentation products}}{\text{Increase of optical density at 415 nm of fermented artificial soymilk}} \times 100 \times \frac{1}{50}\right) \times \text{dilution rate}$$

2.4. Identification of DSW-derived lactic acid bacteria

After thawing DSW-derived lactic acid bacteria stored by freezing as glycerol stocks at −80℃, 16S rRNA gene analysis was performed by a standard method (Fujimoto and Fukui, 2005), and the species was identified. DNA was extracted from the solution using a commercially available gene extraction kit (Takara Bio Inc.) and used as a PCR sample. The PCR product was purified using a commercially available kit (NIPPON Genetics Co., Ltd.), and the resulting DNA was subjected to sequence PCR and used for 16S rRNA gene analysis. Analysis was consigned to the Genome Science Laboratory in our university.

2.5. Growth temperature of DSW-derived lactic acid bacteria

After the DSW-derived strains were thawed like in section 2.4, they were pre-cultured on MRS agar medium at 27℃ for a day. The strains were stationary incubated at 20℃, 37℃, 45℃, and 50℃ for 2 weeks. After incubation, the state of growth was observed with the naked eye. Those in which turbidity was visually observed in the liquid medium were determined to be positive for growth.

2.6. NaCl tolerance of DSW-derived lactic acid bacteria

After serially diluting (10^{-0}–10^{-3}) the suspension, samples of DSW-derived lactic acid bacteria were pre-cultured by the method described in section 2.5 on MRS agar medium with NaCl (0%–8%) added to the separation medium. After 14 days of incubation, the growth of these strains was visually evaluated. Those in which turbidity was visually observed in the liquid medium were determined to be positive for growth.

2.7. Growth pH of DSW-derived lactic acid bacteria

Two hundred μL of each solution of DSW-derived lactic acid bacteria precultured in the same manner was in-

Table 1. Results of 16S rDNA analysis of DSW, SSW and FF-derived lactic acid bacteria

DSW	No. of strain	SSW	No. of strain	FF	No. of strain
Lactoplantibacillus plantarum	18	*Lactiplantibacillus plantarum*	15	*Lactiplantibacillus plantarum*	9
Pediococcus pentosaceus	4	*Lactobacillus derbrueckii*	3	*Levilactobacillus brevis*	3
Leuconostoc mesenteroides	1	*Psiglobus halotolerans*	3	*Lactobacillus sakei*	3
Levilactobacillus brevis	1	*Lactobacillus coryniformis*	2	*Leuconostoc mesenteroides*	2
Pediococcus daussenii	1	*Pediococcus penosaceus*	2	*Leuconostoc lactis*	1
Pediococcus acidilactici	1	*Pediococcus acidilactici*	1	*Lactobacillus senmaizukei*	1
Not identified	6	*Lactobacillus farciminis*	1	Not identified	6
		Leuconostoc mesenteroides	1		
		Lactococcus lactis	1		
		Not identified	5		
Total	32	Total	34	Total	25

oculated into 10 mL of MRS liquid medium. Growth was investigated over a wide pH range (pH 2, 3, 8, 9 and 10). After inoculation, they were cultured for 7 days at 27℃.

2.8. Amount of lactic acid produced by DSW-derived lactic acid bacteria

MRS liquid medium (20 mL) was inoculated with 200 μL of DSW lactic acid bacteria solution that had been pre-cultured for a day at 27℃, and cultured for 3 days at 27℃. After incubation, centrifugation was performed in the same condition of section 2.2. The amount of lactic acid produced was measured by the method described in the literature (Hui *et al.* 2017). In addition, the number of bacteria in the culture solution was measured by turbidity (OD_{630}), and the amount of lactic acid produced per growth of each strain was calculated.

2.9. Statistical analysis

All data were expressed as mean ± SD. Comparisons between multiple groups were performed using the Steel-Dwass test (ANOVA).

3. Results

3.1. Soymilk fermentability and the α-glucosidase inhibitory activity of DSW-derived strains

The fermentability of soymilk was confirmed in 21 out of 32 DSW-derived strains shown in Table 1 (65.6%). SSW-derived strains prepared as one comparative sample suggested the fermentability of soymilk in 30 out of 34 (88.2%) and FF -derived strains prepared as the other comparative sample suggested soymilk fermentation ability in all 25 (100%).

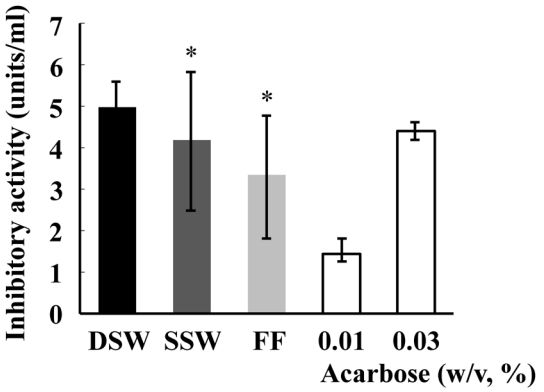

Fig. 2. Comparison of α-glucosidase inhibitory activity of soymilk fermented with lactic acid bacteria isolated from three different sources.
Asterisks indicate significant differences between the following groups. DSW vs SSW, DSW vs FF, SSW vs FF (mean±SD, *$p<0.05$, Steel-Dwass test, ANOVA). Concentrations of acarbose prepared as a positive control for this activity were 0.01 and 0.03 (%,w/v) (n=3, mean±SD).

Owing to the method of section 2.3, the α-glucosidase inhibitory activity of soymilk fermented by DSW-derived lactic acid bacteria were examined and compared with the activity of other-derived isolates. As a result, it was suggested that the activity of soymilk fermented by DSW-derived isolate was higher than the activity of oth-

er-derived isolates such as SSW and FF. Acarbose was prepared to positive control in this test. The inhibitory activity of soymilk fermented by DSW-derived isolates was equivalent to that of 0.03 (%, w/v) acarbose (Fig. 2).

3.2. Identification results of DSW-derived strains

For DSW-derived lactic acid bacteria, the 16S rDNA base sequence (approximately 500 bp) was analyzed, and the homology between the sequences determined using the BLAST method and known base sequences on the database was examined. As a result, high homology (99.0%) or more was found. The strains were judged to be the same species of standard strain of *Lactiplantibacillus plantarum*, which was found to be the most dominant species (Zheng *et al.* 2020). The ratio of *L. plantarum* to the isolates was the highest at 58.0% in DSW-derived isolates, while it was 44.1% in SSW-derived isolates and 36.0% in FF-derived isolates (Table 1).

3.3. Growth temperature of DSW-derived lactic acid bacteria

Good growth was observed in the temperature range of 4 to 40℃ in all isolates. The characteristics of growth of marine environment-derived strains such as DSW and SSW were shown in only higher cultivation temperature range at 40–45℃. FF-derived isolates were never able to grow in cultivation temperature at 50℃.

3.4. NaCl tolerance of DSW-derived lactic acid bacteria

These isolates showed little difference in relative growth rates at NaCl concentrations of 4–7%. However, above 8%, marine-derived strains such as DSW and SSW grew slightly better than FF-derived strains.

3.5. Growth pH of DSW-derived lactic acid bacteria

All strains showed good growth in a wide range of pH 3 to pH 10. On the other hand, at pH 2, DSW-derived isolates grew better than other isolates.

3.6. Lactic acid-producing ability of DSW-derived lactic acid bacteria

The amount of lactic acid produced in soymilk fermented by the DSW isolates was 1.50 ± 0.72 mmol/L. Similarly, the amount of lactic acid produced in soymilk fermented by the SSW-derived isolates was 1.69 ± 1.12 mmol/L and 1.63 ± 0.69 mmol/L for the FF-derived isolates, respectively. There was almost no difference in

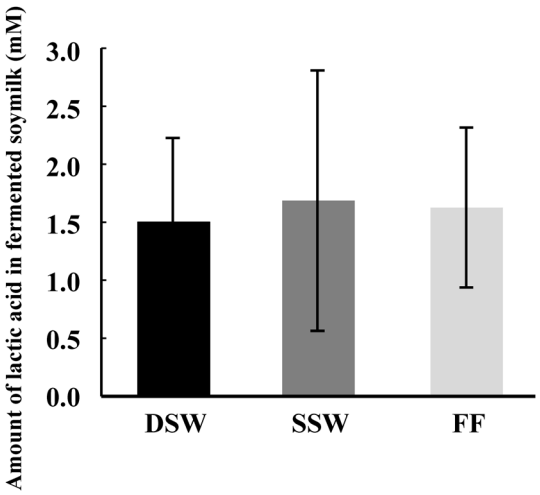

Fig. 3. Comparison of lactic acid content in soymilk fermented with lactic acid bacteria isolated from three different sources.

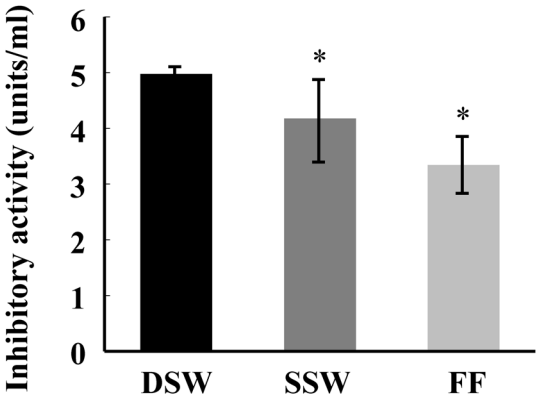

Fig. 4. Comparison of α-glucosidase inhibitory activity of soymilk fermented by *L. plantarum* isolated from three different sources.
Eighteen, 14 and 9 strains of *L. plantarum* were collected from DSW, SSW and FF, respectively. Asterisks indicate significant differences between the following groups. DSW vs SSW, DSW vs FF, SSW vs FF (mean ±SD, *$p < 0.05$, Steel-Dwass test, ANOVA).

the lactic acid-producing ability of the lactic acid bacteria collected from these three sources (Fig. 3).

3.7. Alpha-glucosidase inhibitory activity of soymilk fermented with DSW-derived *L. plantarum*

As in section 2.3, the α-glucosidase inhibitory activity of soymilk fermented with *L. plantarum* derived from DSW was examined and compared with those of other sources. The results suggested that the activity of soymilk fermented with *L. plantarum* derived from DSW was higher than those derived from other source (Fig. 4).

3.8. Lactic acid productivity of DSW-derived *L. plantarum*

The lactic acid production of soymilk fermented with DSW-derived *L. plantarum* was 1.15 ± 0.15 mmol/L. Similarly, the lactic acid production of soymilk fermented with the SSW-derived isolates was 1.42 ± 0.53 mmol/L and 1.55 ± 0.61 mmol/L with the FF-derived isolates, respectively. There was little difference in the lactate productivity of *L. plantarum* collected from the three sources (Fig. 5).

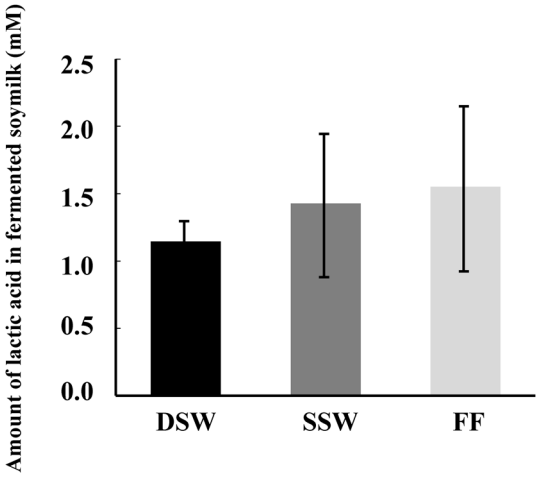

Fig. 5. Comparison of lactic acid content in soymilk fermented with *L. plantarum* isolated from three different sources.
In this study, we collected 18 strains from DSW, 14 strains from SSW, and 9 strains from FF (mean±standard deviation).

4. Discussion

In the present study, lactic acid bacteria isolated from DSW in Izu-Akazawa, Ito City, Shizuoka Prefecture were compared with them isolated from another isolation sources. Considering its use in the field of functional foods, the fermentability and α- glucosidase inhibitory activity of soymilk was investigated. As shown Fig. 2, it was suggested that the α-glucosidase inhibitory activity of soymilk fermented with lactic acid bacteria derived from DSW was the highest among all isolates ($p < 0.05$), however as described above section 3.1, the fermentability of soymilk was lower than that of other isolates. Therefore, there was no significant relationship between soymilk fermentability and α-glucosidase inhibitory activity. From this result, it was necessary to pay attention to α-glucosidase activity rather than the fermentability of fermented soymilk by DSW-derived lactic acid bacteria in this study. Its functionality was considered very useful for those applications derived from DSW. Next, we considered the characteristics of lactic acid bacteria collected from three different sources. The results showed that the proportion of *L. plantarum* in lactobacilli from DSW isolates was much higher than other isolates.

A similar result was also reported as characteristics of lactic acid bacteria isolated from DSW by Yamada *et al.* (2022). On the other hand, Haraguchi *et al.* (2019) reported that the soymilk fermented by *L. plantarum* isolated from child feces was confirmed to have the α-glucosidase inhibitory activity.

Considering these reports, it was suggested as a possibility that the presence of *L. plantarum* among lactic acid bacteria isolated from isolation sources was a very important thing on expression of the α-glucosidase inhibitory activity. This study suggested that there were some α-glucosidase inhibitors produced by *L. plantarum* from soymilk. The DSW strains had the lowest lactate-producing ability compared to the SSW and FF strains, but had the highest α-glucosidase inhibitory activity (Figs 4 and 5).

Soymilk focused in this study is an excellent food containing excellent nutrients such as oligosaccharides, antioxidants, isoflavonoid derivatives and polyamines (Izumi *et al.* 2007; Yamamoto *et al.* 2019). So-called "fermented soymilk" is attracting attention as a functional food because it can impart the nutrients of soymilk and the functionality of lactic acid bacteria, and it is easy to be ingested by the body (Kinoshita *et al.* 2016). This study suggests that the α-glucosidase inhibitory activity of fermented soymilk is comparable to that of acarbose, raising expectations for fermented soymilk with DSW-derived *L. plantarum.*

Recently, it has been well-known that isoflavonoid glycoside such as, daidzin and genistin contained in soymilk are converted into the aglycone type such as, daidzein and genistein by β-glucosidase produced by certain lactic acid bacteria. It has been reported that daidzein and genistein have the α-glucosidase inhibitory activity (Lee and Lee, 2001; Park *et al.* 2013). As mentioned above, the analysis of aglycone isoflavones such as daidzein and genistein in soymilk fermented by *L. plantarum* derived from DSW remains a major issue for future work.

Finally, the characteristics of lactic acid bacteria collected from DSW is discussed. In this study, we identified 91 strains of lactic acid bacteria derived from DSW, SSW, and FF by nucleotide sequence analysis of 16S rDNA. There was great interest, in a microbiological point of view, to investigate why *L. plantarum*, a representative terrestrial plant-derived lactic acid bacterium, was isolated from DSW. There were few differences in *L. plantarum* from each isolation source except for the phenotype in special cultivation conditions such as the growth temperature and pH range. *L. plantarum* is famous for its strong seed and shows high survival rate even in foods with high salt content such as kimchi and fermented rice bran foods (Morichi, 2008). Since the BF used to isolate the DSW strain was left in an environment at a depth of 800 m for about a month, it is considered as a possibility that the collection by BF randomly selected lactic acid bacteria that can survive in the characteristic environment of deep-sea such as low temperature and higher hydrostatic pressure. As shown in a result of this study, it was suggested that DSW is very efficient on isolation of *L. plantarum* among isolation sources. It was also suggested that soymilk fermented with DSW-derived *L. plantarum* strains had the α-glucosidase inhibitory activity and the possibility on applications as a new functional food.

In addition, future research is expected to further clarify the characteristics of DSW-derived lactic acid bacteria.

We would like to continue our multifaceted functional search for DSW-derived lactic acid bacteria.

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