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Short Communication

In vitro antioxidant, anti-glycation, and bile acid-lowering capacity of peanut milk fermented with *Lactiplantibacillus plantarum* Kinko-SU4

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ABSTRACT

Plant-based milk-like products from soybeans and other legumes and nuts have been explored worldwide, owing to their nutritional and functional characteristics. This study was conducted to develop new functional food materials from peanut (*Arachis hypogaea*) milk (PM) with desirable health functions to mitigate lifestyle and age-related diseases. The antioxidant, anti-glycation and bile acid-lowering properties of PM fermented with lactic acid bacteria *Lactiplantibacillus plantarum* Kinko-SU4 (FPM) were determined *in vitro*. *L. plantarum* Kinko-SU4 lowered the pH level from 6.4 to 4.3, 3.9, and 3.7 at 10, 24, and 48 h, respectively. The lactic acid concentration was 4.4 mg/mL after 48 h of incubation. The starter degraded the dissolved proteins in PM, including Ara h 1, one of the peanut allergens. Although the total phenolic content was 36% lower in FPM than in unfermented PM, O_2^- radical-scavenging capacity was high in FPM. Anti-glycation in a bovine serum albumin-fructose model and the bile acid-lowering capacities of PM were distinctly increased following fermentation. The result of this study infers that PM fermented with *L. plantarum* Kinko-SU4 can be considered a desirable food material to prevent and ameliorate chronic lifestyle diseases, particularly in the elderly.

1. Introduction

The aging population has created huge economic burdens and medical problems worldwide, even in developed countries (Nagarajan et al., 2021). Aging increases the risk of developing chronic lifestyle diseases such as diabetes, metabolic disorders, cardiovascular disease, and cancer (Santos and Sinha, 2021). According to the "Annual Report on the Aging Society" by Cabinet Office of Japan in 2020, 29% and 15% of the Japanese population are aged 65 and 75 years or above, respectively. The number of patients with lifestyle-related diseases increases, leading to 60% of the total deaths in this age group and contributing to 30% of the total medical expenses. The risk of viral infections increases with age and is exacerbated by lifestyle-related diseases (Lange, 2021). In addition to improving eating habits and encouraging moderate exercise, the development of functional food products can also help prevent and ameliorate lifestyle-related diseases and strengthen immunity (Sharma, 2021).

The interest in plant-based milk-like products made from soybeans and other legumes and nuts is increasing, owing to their nutritional and functional characteristics (Grossmann et al., 2021). Individuals with health problems such as lactose intolerance, allergies to the proteins in cow's milk, or those with a vegetarian diet can use plant-based milk products (Reyes-Jurado et al., 2021). Peanut *(Arachis hypogaea)*, one of the most popular nuts, are rich sources of lipid (50% w/w), carbohydrate (21%), protein (24%), and other biologically active compounds, such as stilbenoids, flavonoids, and phytosterols (Mingle et al., 2022). Peanut milk (PM) products are also popular worldwide (Jia et al., 2021).

The overproduction of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydroxyl (OH^-) radicals, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), has been associated with developing various chronic and age-related degenerative diseases, such as cancer, respiratory, neurodegenerative, and digestive diseases (Shields et al., 2021). Advanced glycation end-products (AGEs), particularly endogenously produced AGEs, have been linked to chronic hyperglycemia and age-related diseases (Rungratanawanich et al., 2021). Hypercholesterolemia, implicated in cardiovascular diseases, is also a major human health concern (Collado et al., 2021). Food materials with bile acid-lowering (hydrolysis and binding) properties inhibit the reuse of bile acids during enterohepatic circulation, promoting the conversion of bile acids from cholesterol and reducing the amount of cholesterol in the blood (Jia et al., 2021). Therefore, studying food materials with antioxidant, anti-glycation, and bile acid-lowering properties is important.

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(Nishida et al., 2021; Vijaykrishnaraj and Wang, 2021; Mirmiran et al., 2022).

The antioxidant capacity of peanut-oil, -skin, and -hydrolyzed proteins have been reported (Kumar et al., 2021; Yu et al., 2021; Zhang et al., 2022). Although the anti-glycation and hypocholesterolemic properties of peanut skin are also evidenced (Shimizu-Ibuka et al., 2009; Zhao et al., 2021), the activities of whole- or deskinned-peanuts are not well known. Previous studies have shown that the antioxidant (O_2^- scavenging), anti-glycation, and bile acid-lowering properties of some plant food materials, such as white Japanese radish, rice bran, loofah, argan press cake and some edible algae, are increased by lactic acid fermentation (Kuda et al., 2010; Eda et al., 2016). Therefore, this study aimed to investigate the antioxidant, anti-glycation, and bile acid-lowering properties of PM compared with PM fermented with selected lactic acid bacteria (FPM) for developing new functional food materials with desirable nutritional value, health functions, and rheology that can alleviate the problems associated with lifestyle and age-related diseases.

2. Material and method

2.1. PM-preparation and chemicals used

Raw peeled peanut products from China were purchased from a food retail store (Kobe Aarti, Kobe, Japan), and 50 g was soaked in distilled water (DW) overnight. The soaked peanuts were cooked in 500 mL of DW using the "soybean milk mode" of a soybean milk maker (DJ-06P, Fukunou Sangyo, Mitsugi, Japan) and drained using a strainer and kitchen net. In the soybean milk maker, the water and peanut mixture was first boiled for 5 min, crushed for 5 min, and then stirred for 20 min. The slurry was then autoclaved at 115 °C for 15 min to obtain the PM.

Folin-Ciocalteu phenol reagent, phenazine methosulfate (PMS), 3-(2-pyridyl)-5,6-di(p-sulfophenyl)1,2,4-triazine disodium salt (ferrozine), β-nicotinamide adenine dinucleotide (NADH), and nitroblue tetrazolium salt (NBT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Catechin, potassium ferricyanide, trichloroacetic acid, $FeCl_3$, bovine serum albumin (BSA), D-fructose (Fru), bile powder (Oxgall), and deoxycholic acid (DCA) were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). The remaining reagents were of analytical grade.

2.2. PM fermentation with lactic acid bacteria

2.2.1. Screening for starter cultures

A total of 43 lactobacilli and 32 lactococci strains (Table S1) isolated from coastal samples (algal beach casts, beach plants, and sand) (Eda et al., 2016; Kuda et al., 2016a, 2016b) were stored in the laboratory at −80 °C with Microbank beads (Iwaki & Co., Tokyo, Japan) to screen for starters utilized in this study. A bead of each strain was then inoculated in 3 mL of de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) and pre-cultured at 37 °C for 48 h. A loop of the culture was inoculated in 3 mL of PM and incubated at 37 °C for 3 days; thereafter, the pH was measured using a pH meter (22B, Horiba, Kyoto, Japan). The lactobacilli and lactococci strains that generated the lowest pH value were selected for further experiments.

2.2.2. PM fermentation

One selected strain each from lactobacilli and lactococci strains, *Lactiplantibacillus plantarum* Kinko-SU4 (accession no. LC428213), isolated from the flower of *Hydrangea macrophylla*, and *Lactococcus lactis* subsp. *lactis* Oga-SU2 (accession no. LC208001), isolated from beach sands, were pre-incubated in the MRS broth. The pre-culture (1 mL) was inoculated in 100 mL of PM and incubated at 37 °C for 48 h. Viable counts and pH values were measured at 0, 10, 24, and 48 h of incubation using the MRS agar plating method and the pH meter, respectively.

2.3. Chemical analysis of FPM

PM was incubated for 48 h with a selected strain (FPM). PM and FPM were centrifuged at 5000 *g* for 10 min at 4 °C to measure the lactic acid concentration, ultraviolet (UV) absorbance spectrum, protein, ammonia, and total phenolic compound content (TPC).

Lactic acid production was determined by high-performance liquid chromatography (HPLC) on an ICSep ICE-ORH-801 column (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (Xia et al., 2022). The protein content was measured using the Qubit Protein Assay Kit (Thermo Fisher Scientific, Tokyo, Japan), and the molecular weight pattern of the protein was measured with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using Compact PAGE (ATTO, Tokyo, Japan) and 5–20% polyacrylamide gels (e-PAGEL, ATTO) according to the manufacturer's instructions. TPC was measured with Folin-Ciocalteu phenol reagent, as previously reported by Eda et al. (2016).

The UV spectra were measured using a UV–visible spectrometer (ES-2, Tokyo, Japan). The relative viscosities of PM and FPM were directly determined by an oscillation viscometer (Viscomate VM-1G; Yamaichi Electronics; Osaka, Japan) under ice-cold conditions (Takei et al., 2017). The relative viscosity was then calculated as the quotient of the AES viscosity divided by the viscosity of DW. The color parameters (L^*, a^*, b^*) were measured with a spectrophotometer (NF333; Nippon Denshoku Industries, Tokyo, Japan) (Shibayama et al., 2019).

2.4. Antioxidant properties

2.4.1. O_2^- radical-scavenging activity

The scavenging capacity was determined by the O_2^- radical (the first ROS to be generated in the body) and was measured using the non-enzymatic NBT method as previously reported (Kaga et al., 2021). Serially diluted samples (0.1 mL) were added to a 96-well microplate ($n = 3$) with 0.025 mL of 0.25 mol/L phosphate buffer (pH 7.2), 0.025 mL of 2 mmol/L NADH, and 0.025 mL of 0.5 mmol/L NBT. The absorbance was measured at 560 nm (Abs1) using a microplate reader (SH-1000 Lab, Corona Electric, Hitachinaka, Japan). This was followed by the addition of 0.025 mL of 0.03 mmol/L PMS. The mixture was incubated in the dark at 22 °C for 5 min, and the absorbance was measured at 560 nm (Abs2). The radical-scavenging capacity was calculated using the following formula (1):

$$\text{Radical-scavenging capacity } (\%) = \left(1 - \frac{\text{Abs2 : sample} - \text{Abs1 : sample}}{\text{Abs2 : control} - \text{Abs1 : control}}\right) \times 100 \quad (1)$$

Using the concentration-dependent curve of the sample and catechin solutions, the antioxidant capacities were expressed as catechin equivalent (CatEq.)/mL.

2.4.2. Ferric-reducing power

To estimate the reducing power of the samples, the Fe-reducing power was measured using a previously described method (Kaga et al., 2021); 0.05 mL of serially diluted samples and DW (control) were added to a 96-well microplate ($n = 3$), with 0.25 mL of 0.1 mol/L phosphate buffer (pH 7.2) and 0.025 mL of 1% (w/v) potassium ferricyanide, and incubated at 37 °C for 60 min. Then, 0.025 mL of 10% (w/v) trichloroacetic acid and 0.1 mL DW were added. The absorbance was measured at 700 nm (Abs1), 0.025 mL of 0.1% (w/v) $FeCl_3$ was added, and the absorbance was measured again at 700 nm (Abs2). The Fe-reducing power was calculated as follows (2):

$$\text{Redusing power (Absorbances at 700 nm)} = (\text{Abs2 : sample} - \text{Abs1 : sample}) - (\text{Abs2 : control} - \text{Abs1 : control}) \quad (2)$$

2.5. Anti-glycation properties

The anti-glycation activity in the BSA-Fru models was determined as previously described (Eda et al., 2016). Fru (1.5 mol/L, 0.5 mL) was mixed with 0.5 mL samples and 0.5 mL sodium phosphate buffer [50 mmol, pH 7.4, with 0.02% (w/v) sodium azide] in screw-capped test tubes, followed by incubation at 37 °C for 2 h. BSA (30 mg/mL, 0.5 mL) was added to each test tube, and the mixtures were incubated at 37 °C for 5 days. Fluorescence excitation was measured at 340 nm and emission at 420 nm using a multiple microplate reader (SH-9000; Corona Electric). The percentage of AGE inhibition was calculated using the following equation (3):

$$\text{Anti-glycation } (\%) = \left(1 - \frac{\text{Fl 5d sample} - \text{Fl 0d sample}}{\text{Fl 5d control} - \text{Fl 0d control}}\right) \times 100 \quad (3)$$

where Fl 0d and Fl 5d represent the fluorescence intensity after reaction for 0 and 5 days, respectively.

2.6. Bile acid-lowering activity

PM and FOM samples were mixed with three times the volume of ethanol and centrifuged at 5000 *g* for 10 min at 4 °C. The resulting pellet was dried for brewing at 20 °C for 4 h in a safety cabinet (BHC-1307IIA2, Airtech Japan, Tokyo, Japan). 0.1 g of the samples were suspended in 10 mL of phosphate-buffered saline (PBS, pH 7.2; Nissui Pharmaceutical, Tokyo, Japan). The suspension (0.5 mL) was added to the same volume of PBS containing 0.5 mg/mL bile or 1.5 mmol/L cholic acid and incubated for 30 min at 37 °C under a shaker (130 rpm). The mixture was then centrifuged (3000 *g* for 10 min at 4 °C), and the amount of bile acid remaining in the supernatant was determined using a commercial kit (TBA Test Wako, FUJIFILM Wako Pure Chemical) according to the manufacturer's protocol.

2.7. Statistical analysis

Measured values ($n = 3$) are presented as mean ± standard error of the mean (SEM). To compare the CFU and pH values in PM fermentation test, a one-way analysis of variance was performed, and the individual means were compared using Tukey's test. To compare differences in the antioxidant, anti-glycation, and bile acid-lowering capacities among groups, the individual means were compared using the Student's *t*-test. All statistical analyses were performed using statistical software (Excel Statistic Ver. 6; Esumi, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. FPM properties with lactic acid bacteria

3.1.1. Starter selection

Approximately 520 mL of PM was prepared from 50 g of dried peanut seeds. PM contained 93% (w/v) water, and the dried residue weighed 94 g. Using the PM fermentation screening test, *L. plantarum* Kinko-SU4 and *L. lactis* Oga-SU2 were selected from 75 strains. The selected strains lowered the pH from 6.8 to 3.8 following incubation (Table S1).

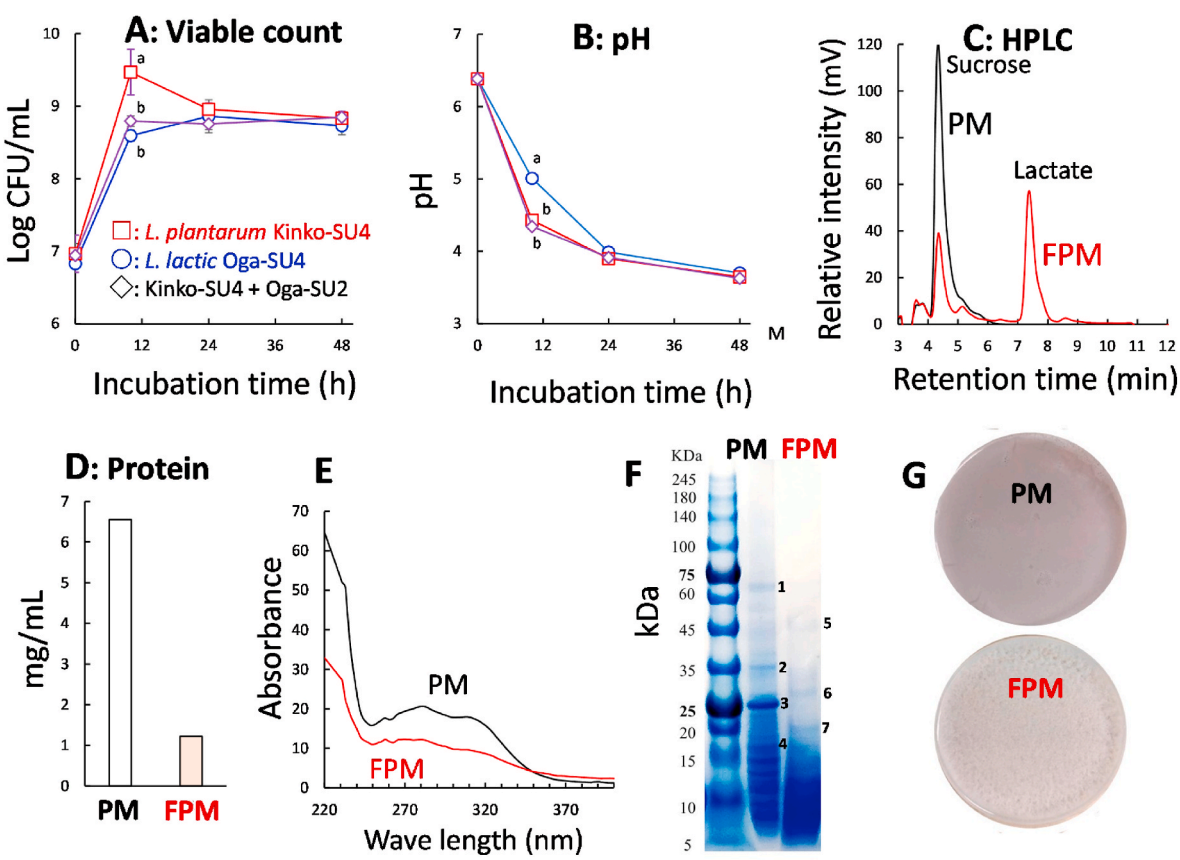

Fig. 1. Colony-forming unit (CFU; A), pH change (B), high-performance liquid chromatography (C), dissolved protein content (D), ultraviolet-absorption spectrum (E), and sodium dodecyl sulfate-polyacrylamide gel image appearance (F) of peanut milk (PM) and PM fermented with *Lactiplantibacillus plantarum* Kinko-SU4 at 37 °C for 48 h (FPM). Values in A and B are mean ± standard error of the mean (SEM) ($n = 3$). [a, b] Values with different letters indicate significant differences at $p < 0.05$.

3.1.2. Lactic acid fermentation with selected starters

The viable counts of *L. plantarum* Kinko-SU4 and *L. lactis* Oga-SU2 in PM increased from approximately 7 log colony-forming units (CFU)/mL to 9.4 and 8.6 log CFU/mL after 10 h of incubation (Fig. 1A). The pH values of *L. plantarum* Kinko-SU4 and *L. lactis* Oga-SU2 lowered immediately from 6.4 to 4.5 and 5.0, respectively, after 10 h of incubation (Fig. 1B). The strains had no synergistic effect. From these results, *L. plantarum* Kinko-SU4 was selected as the FPM starter in subsequent experiments for simple and stable fermentation. During the 48 h of fermentation with *L. plantarum* Kinko-SU4, lactic acid (13.8 μmol/mL), mainly sucrose, was generated from saccharides in the PM (Fig. 1C).

3.2. Dissolved protein and related compounds

The soluble protein contents in PM and FPM were approximately 6.55 and 1.22 mg/mL, respectively (Fig. 1D). The UV absorbance spectra of PM and FPM were consistent with the protein concentrations (Fig. 1E). The SDS-PAGE band patterns of the samples are shown in Fig. 1F. Four major bands (bands 1–4 in Fig. 1F) in PM disappeared following fermentation, while additional bands (bands 5–7) appeared.

According to Li et al. (2013), bands 1 and 4 plausibly correspond to major peanut allergens Ara h 1 and Ara h 2, respectively. Band 3 was estimated as pyrolysis products. Recently, Ara h1 degradation capacity of a selected *L. plantarum* strain, similar to *Bacillus subtilis* natto, has been reported (Pi et al., 2022). The strains used in this experiment may also be used for allergen-reduced foods.

3.3. Colour and viscosity of FPM

The difference in appearance between PM and FPM was observable with the naked eye (Fig. 1G). As shown in Table 1, reddish (a^*) and yellowish (b^*) pigmentation was lowered by the fermentation. Additionally, little agglomeration was observed due to the fermentation. The relative viscosity of PM and FPM were 3.23 and 2.16, respectively.

Although the pigment in the peanut skin is thought to contain polyphenols, such as resveratrol, as functional materials (Bansole et al., 2012), the pale color after fermentation may be easier to use for milk type products. The decrease in viscosity might have involved the decomposition of the major components and changes in the three-dimensional structure.

3.4. TPC and antioxidant properties

Fermentation decreased the TPC of PM from 0.96 to 0.61 μmol CatEq./mL (Table 1). However, O_2^- radical-scavenging activities were significantly increased from a non-detectable lower capacity to 3.3 μmol CatEq./mL (Fig. 2A). In contrast, Fe-reducing power was three folds decreased by the fermentation, though the activity of the intact PM was not so high (0.17 μmol CatEq./mL, Fig. 2B).

TPC is regarded as an active compound with reducing power and the capacity for radical-scavenging in various plant foods, including peanuts. Although increased TPC and antioxidant capacities in deskinned PM by fermentation had been reported (Bensmira and Jiang, 2015), these capacities are higher in peanut skin than in deskinned seeds (Kumar et al., 2021). Moreover, TPC and Fe-reducing power decreased due to fermentation; however, the O_2^- radical-scavenging activity increased in the present study. These results are consistent with previous studies about fermented plant products (Guo et al., 2019; Kaga et al., 2021). O_2^- radical-scavenging capacity of lactate and various peptides has been reported (Groussard et al., 2000; Esfandi et al., 2019). The increase of O_2^- radical-scavenging capacity may involve the formation of lactate shown in Fig. 1C, and the protein-derived low molecular weight substances shown in Fig. 1D–F.

In most organisms, O_2^- radicals are converted to H_2O_2 by superoxide dismutase, and H_2O_2 is stable in the absence of transition metal ions (Pisochi et al., 2021). However, OH^- radicals can be formed by the reaction of superoxide with H_2O_2 in the presence of metal ions, particularly ferrous or copper ions. OH^- radicals are more reactive (and toxic) than O_2^-. Therefore, the high O_2^- radical-scavenging capacity of the FPM indicated the capacity to decrease the levels of O_2^-, H_2O_2, and OH^- radicals.

Table 1
Total phenolic content, relative viscosity, and colour indices of peanut milk and fermented peanut milk.

		Peanut milk	Fermented peanut milk
Total phenolic content (μmol CatEq./mL)		0.958 ± 0.006	0.610 ± 0.047*
Relative viscosity		3.23 ± 0.01	2.16 ± 0.03**
Colour indices	L^*	26.82 ± 0.51	25.19 ± 0.55
	a^*	3.13 ± 1.57	−15.7 ± 1.03**
	b^*	4.53 ± 1.06	1.31 ± 0.44*

Values are mean ± standard error of the mean ($n = 3$).
*$p < 0.05$, **$p < 0.01$.

3.5. Anti-glycation properties

The activity of 125–500 μL/mL samples in the BSA-Fru model is shown in Fig. 2C. The concentration-dependent activity can be seen even in PM (36–65%) and increases significantly to 52–91% inhibition as a result of FPM. Albumin is abundant in serum and can be glycated at multiple sites, particularly in patients with diabetes mellitus (Qiu et al., 2021). The effect of the extracts on all protein glycation stages was determined by the BSA-Fru model system (Wang et al., 2011). The increase in anti-glycation properties of the BSA-Fru model with increased O_2^- radical-scavenging capacity has been shown in previous studies (Taniguchi et al., 2019; Kaga et al., 2021). Research has shown that scavengers for free radicals, particularly in hydroxyl and O_2^- radicals, inhibit the initial process in the AGEs-producing pathway (Anwer et al., 2021).

3.6. Bile acid-lowering capacities

As shown in Fig. 2D, with 0.2 g/mL of sample, an increase in the bile acid-lowering capacity as a result of fermentation was observed in the Ox-gall (from 0 to 5%), glycocholic acid (GCA) (from 0 to 8%), and DCA (from 3 to 21%) models, although these inhibition percentages were not extremely high. Approximately 70% of cholesterol in the human body is biosynthesized in the liver (Kriaa et al., 2019). From the cholesterol, bile acids are synthesized in the liver (Chiang and Ferrell, 2019). When lesser bile acids are reabsorbed from the intestine in the enterohepatic circulation system, the consumption of cholesterol (the material of bile) is greater, and blood cholesterol levels are also reduced (Stellaard and Lütjohann, 2021). Some probiotic strains, including *L. plantarum,* have low cholesterol-lowering effects as a result of bile acid hydrolase activity (Guo et al., 2019; Huang et al., 2019). In addition, this cholesterol-lowering effect is attributed to bile acid-binding by *L. plantarum* putative probiotic cells (Kuda et al., 2016a, 2016b). The latter property was retained even after heat-sterilizing and showed a lowering effect on plasma cholesterol levels in mice. In this study, the DCA-lowering capacity of FPM was clear. Secondary bile acids, including DCA, have been thought to be correlated with carcinogenesis combined with gut microbiota metabolism (Liu et al., 2020).

Furthermore, this study revealed that *L. plantarum* Kinko-SU4 could ferment PM properly. TPC was reduced by fermentation; therefore, the increased O_2^- radical-scavenging, anti-glycation, and DCA-lowering properties may correlate with organic acids, protein metabolites, and cell membrane. FPM can be considered a desirable food product to prevent and ameliorate chronic lifestyle diseases, particularly in the elderly. Future studies are required to analyze free amino acid composition, sensory tests, and the safety and functional properties of FPM *in*

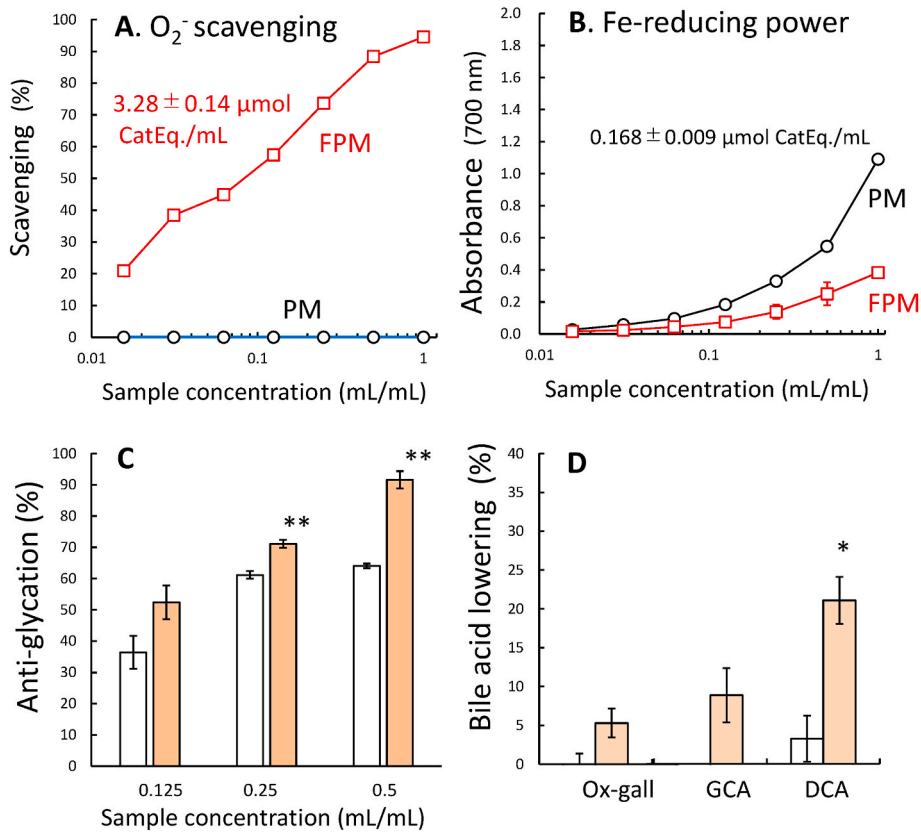

Fig. 2. Superoxide anion radical-scavenging (A), Fe-reducing power (B), anti-glycation in BSA-fructose model (C), and bile acid-lowering (D) capacities of peanut milk (PM: Circles and open columns) and PM fermented with *Lactiplantibacillus plantarum* Kinko-SU4 at 37 °C for 48 h (FPM: Squares and closed columns). Values are mean ± standard error of mean (SEM) ($n = 3$). *** Values with different superscript letters indicate significant differences (*$p < 0.05$, **$p < 0.01$).

vitro and *in vivo* for the application of FPM in processed foods. Additionally, they should be focused on establishing an association between the composition parameters in FPM and the bioactivities shown in this study.

4. Conclusion

The antioxidant, anti-glycation, and bile acid-lowering properties of both PM and FPM were determined to develop new functional food materials with desirable nutritional value, health functions, and rheology that can inhibit lifestyle and age-related diseases. *L. plantarum* Kinko-SU4 was found to ferment PM properly, and the dissolved PM proteins were degraded by the starter strains. The O_2^- radical-scavenging, anti-glycation in the BSA-Fru model, and DCA-lowering capacities of PM were increased by fermentation. Based on our findings, FPM can be considered a beneficial food material that have potentials prevent and ameliorate capacities on lifestyle diseases, particularly in the elderly.

CRediT authorship contribution statement

Mahiro Yamamoto: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Natsumi Handa:** Investigation. **Ayaka Nakamura:** Investigation. **Hajime Takahashi:** Conceptualization, Methodology, Supervision. **Takashi Kuda:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.06.003.

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