



# In vitro antioxidant, anti-glycation and immunomodulation activities of fermented blue-green algae *Aphanizomenon flos-aquae*

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## Abstract

To clarify the antioxidant, anti-glycation and immunomodulatory capacities of fermented blue-green algae *Aphanizomenon flos-aquae* (AFA), hot aqueous extract suspensions made from 10% AFA were fermented by *Lactobacillus plantarum* AN7 and *Lactococcus lactis* subsp. *lactis* Kushiro-L2 strains isolated from a coastal region of Japan. The DPPH and O<sub>2</sub><sup>-</sup> radical scavenging capacities and Fe-reducing power were increased in the fermented AFA. The increased DPPH radical scavenging capacity of the fermented AFA was fractionated to mainly < 3 kDa and 30–100 kDa. The increased O<sub>2</sub><sup>-</sup> radical scavenging capacities were fractionated to mainly < 3 kDa. Anti-glycation activity in BSA-fructose model rather than BSA-methylglyoxal model was increased by the fermentation. The increased anti-glycation activity was fractionated to mainly 30–100 kDa. The NO concentration in the murine macrophage RAW264.7 culture media was high with the fermented AFA. The increased immunomodulation capacity was also fractionated to mainly 30–100 kDa. These results suggest that the fermented AFA is a more useful material for health foods and supplements.

**Keywords** Blue-green algae · *Aphanizomenon flos-aquae* · Fermentation · Antioxidant · Anti-glycation · RAW264.7 cells

## Introduction

A large quantity of blue-green algae, the cyanobacterium *Aphanizomenon flos-aquae* (AFA) inhabits Upper Klamath Lake in south-central Oregon in the United States [1]. AFA is rich in polyunsaturated fatty acids, proteins, vitamins, and minerals [2]. Additionally, the hypocholesterolaemia and anti-diabetes properties of AFA have been reported [3]. Although AFA inhabiting other regions is regarded as undesirable microalgae due to its toxins (such as aphanotoxins), AFA from Upper Klamath Lake does not have toxins and is used for human health food supplements [4].

Reactive oxygen species (ROS), such as superoxide anion radicals (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals,

and singlet oxygen, are generated in the bodies of living organisms [5]. These oxygen species react with important cell components, such as DNA, proteins, lipids, and small cellular molecules, and induce a wide range of common diseases and age-related degenerative conditions [6, 7]. A correlation between ROS and age-related diseases, such as cardiovascular disease, inflammatory conditions, and neurodegenerative diseases, such as Alzheimer's disease and cancer, has been reported [8, 9]. To prevent diseases and aging caused by ROS, the antioxidative capacities of various foods have been studied [10].

Glycation is a non-enzymatic reaction of reducing sugars with amino acids and/or proteins in processed food (Maillard reaction) and *in vivo* [11, 12]. Advanced glycation end products (AGE), such as carboxymethyl lysine and carboxylethyl lysine are generated irreversibly after various intermediates such as glyoxal, methylglyoxal (MGO), and 3-deoxyglucosone [13]. AGEs are thought to induce diabetes and other diabetes- and age-related illnesses such as retinopathy, cataracts, arteriosclerosis, and renal dysfunction [14]. Due to the AGE synthesis contain oxidation process, already there are reports about food materials have both antioxidant and anti-glycation activities with some phenolic compounds [15].

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Nitric oxide (NO) is an ROS that is an endogenously synthesised free radical and a member of the gaseous signalling molecules widely known as gasotransmitters [16]. NO directly modifies its intracellular targets due to its ability to passively permeate the cellular membrane, and it plays important roles in inflammation, life-style, neurological and age-related diseases as mentioned above [17, 18]. The anti-inflammatory effects of various plant foods and ethnic medicinal plants and their chemical compounds were evaluated by the inhibitory effect on NO secretion induced by *Escherichia coli* lipopolysaccharide (LPS) in murine RAW264.7 macrophages [18, 19]. Alternatively, stimulatory effects on NO production have been reported in other food materials, particularly in exopolysaccharide (EPS) containing probiotic lactic acid bacteria (LAB), polyunsaturated fatty acids, and oligo- and poly-saccharides in RAW264.7 cells [20–22].

The antioxidant properties of AFA have been reported [23–25]. The immunomodulation capacities of AFA also have been reported; though, the mechanisms are still not clear [26, 27]. Some LABs isolated from coastal regions can increase the antioxidant ( $O_2^-$  radical scavenging, and protection of macrophages and epithelial-like cells against  $H_2O_2$ ), anti-inflammation, and anti-glycation capacities of suspensions and/or aqueous extract solutions of some edible algae during fermentation [28–31].

In the present study, we sought to clarify the effect of LAB fermentation on the antioxidant, anti-glycation and immunomodulatory capacities of AFA. Hot aqueous extract suspensions made from dry-powdered AFA were fermented by *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis* strains isolated from a coastal region in Japan. These LAB strains showed higher stress (acid, bile and salinity) resistances compared with ones of their type strain [19, 32]. Then, the in vitro antioxidant and anti-glycation properties and immunomodulation effects on NO secretion from RAW264.7 cells of the non-fermented and fermented AFA suspensions were determined.

## Materials and methods

### Chemicals

(+)-Catechin, Folin–Ciocalteu phenol reagent, the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical phenazine methosulphate (PMS), 3-(2-pyridyl)-5,6-di(*p*-sulfophenyl)1,2,4-triazine disodium salt (ferrozine),  $\beta$ -nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium salt (NBT), methylglyoxal (MGO), and lipopolysaccharide (LPS) from *Escherichia coli* O111 B4 were purchased from Sigma-Aldrich (St. Louis, MO). Potassium ferricyanide, trichloroacetic acid (TCA), bovine serum albumin

(BSA), D-fructose (Fru) and Griess-Romijn nitrite reagent were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan).

Freeze-dried powder of *Aphanizomenon flos-aquae* (AFA) harvested from Upper Klamath Lake was obtained from Dr's Choice Co., Tokyo, Japan. The other reagents were of analytical grade.

### Screening of AFA fermentative lactic acid bacteria

AFA (5 g) was suspended with 200 mL of distilled water (DW) and autoclaved (121 °C for 15 min, 2.5% AFA-S). Twenty-nine *Lactobacillus plantarum* and 10 *Lactococcus lactis* subsp. *lactis* isolated from coastal regions [19, 28, 31, 33] were pre-incubated in 3 mL of de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. Then, 0.03 mL of the pre-culture was inoculated into 3 mL of the 2.5% AFA-S. After 4 days fermentation at 37 °C, the pH value was measured using a pH meter (LAQUA Twin B-711, Horiba, Kyoto, Japan). Strains of *L. plantarum* and *L. lactis* that showed the lowest pH values were selected as starters for further experiments.

### Fermentation of 10% AFA-S

AFA powder (20 g) was suspended with 200 mL of DW, the pH was adjusted to 7.0 with 0.1 mol/mL NaOH, and the suspension was autoclaved (10% AFA-S). 5 mL of 10% AFA-S (5 mL) was fermented by inoculation of the pre-culture (0.05 mL) of the two selected LAB strains. After 0, 1, 2, 4, and 7 days fermentation, the pH values and viable counts with MRS agar plates were determined.

To analyse glucose, lactic acid, and acetic acid in the 7 day fermented 10% AFA-S by high-performance liquid chromatography (HPLC), the sample was centrifuged at  $3000\times g$  at 4 °C for 5 min and the supernatant was filtered using a 0.2  $\mu$ m pore filter. The HPLC conditions were as follows: column, IC9ep ICE-ORH-801 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); operating temperature, 35 °C; elution, 0.005 mol/L of sulfuric acid ( $H_2SO_4$ ); flow rate, 0.8 mL/min. Eluted compounds were detected by a refractive index (RI) detector.

Among the major minerals (Na, K, Ca, Mg, Fe, Cu, and Mn), K, Ca, Fe, Cu, and Mn were measured using commercially available kits from the series of Reagent Set for Water Analyzer (No. 36, No. 48, No. 41B, No. 50, and No. 28, respectively, Kyoritsu Chemical-Check Lab., Corp., Tokyo, Japan). Na was measured using a Na-ion meter (LAQUA Twin B-721, Horiba, Kyoto, Tokyo). Mg was determined using a commercial diagnosis kit (Magnesium B-Test Wako, FUJIFILM Wako Pure Chemical).

After diluting the sample solution with nine volumes of distilled water, the free amino acid content was determined

by an amino acid analyser (L-8900, Hitachi, Tokyo, Japan), as previously reported [34]. This high-performance liquid chromatography (HPLC) analysis method can also detect some amino-acid-related compounds, such as ammonia, ethanolamine, and aminobutyric acids.

### Total phenolic compound content (TPC) of fermented 10% AFA-S

For experiments to determine phenolic content and antioxidant properties, intact and fermented 10% AFA-S was centrifuged at  $3000 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 10 min. The experiments to determine TPC, antioxidant properties, and anti-glycation properties were conducted in triplicate.

Then, 0.03 mL of a diluted supernatant and 0.06 mL of 10% Folin–Ciocalteu solution were placed in a 96-well microplate. After 3 min, 0.12 mL of 10% sodium carbonate was added. The mixture stood for 60 min at ambient temperature and the absorbance was measured at 750 nm using a grating microplate reader (SH-1000 Lab; Corona Electric, Hitachinaka; Ibaraki, Japan). The phenolic content is expressed as catechin equivalents (CatEq)/g dry sample.

### DPPH radical-scavenging capacity

Sample diluted solution (0.1 mL) and ethanol (0.1 mL) were put into a 96-well microplate and absorbance at 517 nm (Abs1) was measured using the microplate reader. Next, 1 mmol/L DPPH radical was added and incubated at  $37\text{ }^{\circ}\text{C}$  for 30 min and the absorbance (Abs2) was measured again. The DPPH radical-scavenging capacity was calculated using the following formula:

$$\text{Radical scavenging capacity (\%)} = (1 - (\text{Abs2 of sample} - \text{Abs1 of sample}) / (\text{Abs2 of control} - \text{Abs1 of control})) \times 100$$

### $\text{O}_2^-$ radical-scavenging capacity

The sample solution (0.1 mL) was treated with 0.05 mL of 250 mmol/L phosphate buffer (pH 7.2), 2 mmol/L NADH (0.025 mL), and 0.5 mmol/L NBT (0.025 mL), while the absorbance at 560 nm was measured as a blank value. After 5 min incubation at room temperature ( $20\text{--}24\text{ }^{\circ}\text{C}$ ) with 0.025 mL of 0.03 mmol/L PMS, the absorbance was measured again. The radical-scavenging capacity was calculated using the above formula.

### Ferric-reducing power

For each 0.05 mL of the sample solution, 0.025 mL of 0.1 mol/L phosphate buffer (pH 7.2) and 0.025 mL of 10 g/L

potassium ferricyanide were placed in a 96-well microplate. After incubation at  $37\text{ }^{\circ}\text{C}$  for 60 min, 0.025 mL of 10% TCA and 0.1 mL of distilled water were added, and the absorbance was measured at 700 nm (Abs1). Next, 0.025 mL of 0.1%  $\text{FeCl}_3$  was added to the mixture and the absorbance was measured again (Abs2). The ferrous-reducing power was calculated using the following formula:

$$\begin{aligned} \text{Reducing power (OD 700 nm)} \\ &= (\text{Abs2 of sample} - \text{Abs1 of sample}) \\ &\quad - (\text{Abs2 of control} - \text{Abs1 of control}) \end{aligned}$$

### Anti-glycation property

The anti-glycation assays in the BSA-Fru and BSA-MGO models were determined using the method of [35] with slight modification [28]. BSA-Fru and BSA-MGO models evaluate all stages and the middle stage, respectively, of protein glycation. 1.5 mol/L Fru or 60 mmol/L (0.5 mL) was mixed with 0.5 mL of AES and 0.5 mL of sodium phosphate buffer (50 mmol/L, pH 7.4, with 0.02% sodium azide) in screw-capped test tubes and kept at  $37\text{ }^{\circ}\text{C}$  for 2 h. BSA (30 mg/mL, 0.5 mL) was added to each test tube, and the mixtures were incubated at  $37\text{ }^{\circ}\text{C}$  for 5 days. Fluorescent AGEs were monitored on a multiple microplate reader (SH-9000; Corona Electric) using 340 nm as the excitation wavelength and 420 nm (for BSA-Fru) or 380 nm (for BSA-MGO) as the emission wavelength. Percentage of the AGE inhibition was calculated by the following equation:

$$\begin{aligned} \text{Anti-glycation capacity (\%)} \\ &= (1 - (\text{F5d of sample} - \text{F0d of sample}) / \\ &\quad (\text{F5d of blank} - \text{F0d of blank})) \times 100 \end{aligned}$$

F0 and F5d represent fluorescent intensity after the reaction for 0 and 5 days, respectively.

### Effects on nitric oxide (NO) secretion by RAW264.7 murine macrophages

The sample suspensions were heated in boiling water for 20 min. After cooling, the suspensions were 1/2 serial-diluted with Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 5% v/v foetal bovine serum (FBS). To determine the immune stimulation and anti-inflammation properties, murine macrophage like RAW264.7 cells (TIB-71; American Type Culture Collection, Manassas, VA) were used [36].

RAW264.7 cells were suspended in the aforementioned medium (6 log cells/mL) and seeded into a 96-well microplate (0.1 mL/well). After incubation at  $37\text{ }^{\circ}\text{C}$  for 2 h in an atmosphere of 5%  $\text{CO}_2$ , the medium was replaced with

fresh medium (0.1 mL) and the sample containing medium (0.1 mL) was added. After a 20 h incubation, the nitric oxide (NO) concentration in the cultured medium was determined with 10% (w/v) Griess-Romijn nitrite reagent as described previously (Shikano et al., 2018). To confirm the cell toxicity, after the incubation, the viability of the RAW264.7 cells with and without the samples were determined with a commercial kit containing a reducing indicator: WST-8 (Cell Counting Kit-8, Dojindo Laboratories, Mashiki, Japan), according to the manual.

For the anti-inflammation assay, LPS solution (4 µg/mL) was added 2 h after the addition of the sample containing medium (0.01 mL/well for a final concentration of 0.19 µg/mL). The sample was incubated for 18 h, and the NO concentration was measured.

### Measuring the TPC, antioxidant capacity, anti-glycation activity and induction capacity for the NO secretion of RAW264.7 cells of the ultrafiltered fractions of the intact and fermented 10% AFA-S

Portions (20 mL) of the supernatant of the intact and fermented 10% AFA-S were separated into six fractions (based on their molecular weights: > 300, 100–300, 30–100, 10–30, 3–10, and < 3 kDa), using an ultrafiltration system VIVASPIN 20 (Sartorius AG, Gottingen, Germany) [19]. The TPC, antioxidant capacities, and NO secretion in the

culture of RAW264.7 cells were measured using the methods described above.

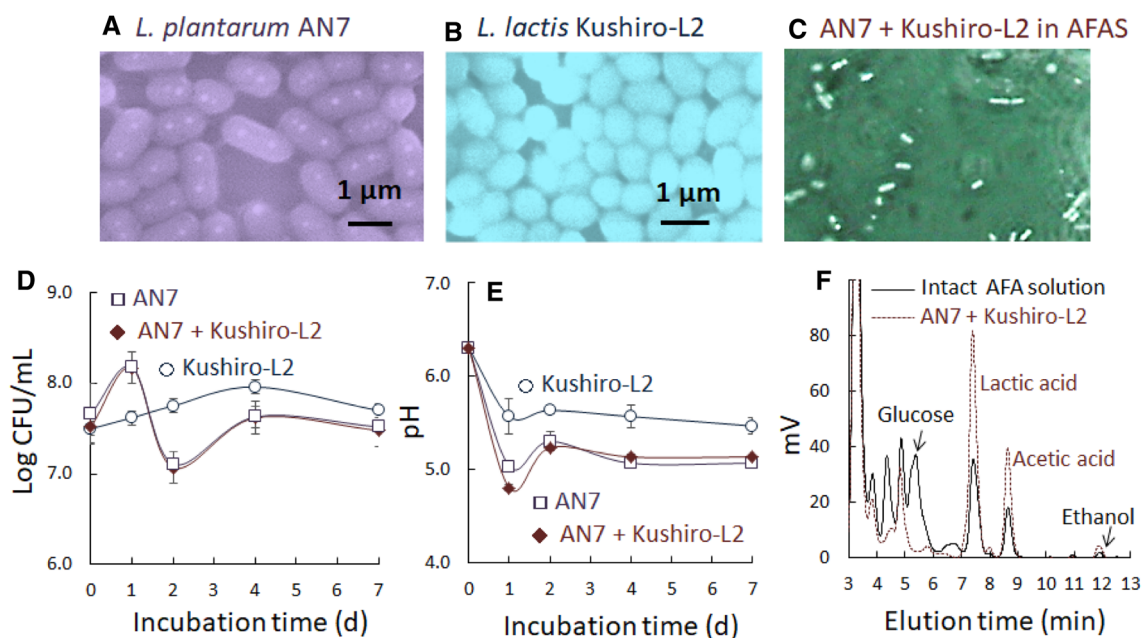
### Statistical analysis

Measured values ( $n = 3$ ) are presented as mean  $\pm$  standard error of the mean (SEM). One-way ANOVA was performed to assess differences among groups, and individual means were compared by Tukey's post hoc test or Student's  $t$ -test, using statistical software (Excel Statistic Ver. 6, Esumi, Tokyo, Japan). Significant differences were accepted at  $p < 0.05$ .

## Results and discussion

### Selected LAB strains

Among the 29 *Lactobacillus plantarum* stocked strains, *L. plantarum* AN7 (Accession No: LC384876, Fig. 1A) isolated from traditional fermented horse mackerel with rice made in Noto Peninsula, Ishikawa [32] showed the clearest pH lowering in 2.5% AFA-S, from 6.3 to 5.1. Among the 10 *Lactococcus lactis* subsp. *lactis* strains, *L. lactis* Kushiro-L2 (Accession No: LC333954, Fig. 1B) isolated from Shirarutoroko Lake, Hokkaido in the present study,



**Fig. 1** Images of *Lactobacillus plantarum* AN7 (A) and *Lactococcus lactis* subsp. *lactis* Kushiro-L2 (B) with a table top SEM (TM3030) and the cells in a 10% (w/v) *Aphanizomenon flos-aquae* suspension (AFA-S) observed under a phase-contrast microscope (C). **D** and **F**:

Changes of the viable counts of the strains and pH, respectively, of the 10% AFA-S during 7 days fermentation at 37 °C. Values are mean and SEM ( $n = 3$ ). **F**: HPLC chromatograph of 7 day fermented 10% AFA-S

showed the clearest pH lowering from 6.3 to 5.6. These two LAB strains were selected for further experiments.

### Fermentation properties

After 7 days incubation of the selected LAB strains in 10% AFA-S, both LAB cells could be observed under a phase-contrast microscope (Fig. 1C). As shown in Fig. 1D, the inoculated *L. plantarum* AN7 increased immediately in 24 h and then decreased. Along with viable count, the pH value decreased in 24 h from 6.3 to 4.8, and then it was 5.1 at 7 days fermentation (Fig. 1E). In contrast, the

inoculated *L. lactis* Kushiro-L2 increased slowly and the pH was lowered to 5.5.

By the HPLC analysis (Fig. 1F; Table 1), glucose and other some saccharides were detected in the intact 10% AFA-S and these were converted to mainly lactic acid, followed by acetic acid and a small amount of ethanol. These organic acid generations were high with *L. plantarum* AN7 rather than *L. lactis* Kushiro-L2. The concentration of the 10% AFA-S fermented with both LAB strains were similar with ones of *L. plantarum* AN7 fermented 10% AFA-S.

These results indicate that *L. plantarum* AN7 can typical lactic acid fermentation in 10% AFA-S, though the pH

**Table 1** Glucose, lactic acid, acetic acid, major minerals and free amino acids in *Aphanizomenon flos-aquae* suspension fermented with lactic acid bacteria

Minerals	Control	<i>L. plantarum</i> AN7	<i>L. lactis</i> Kushiro-L2	AN7 + Kushiro-L2
Glucose and organic acids (mg/mL)*				
Glucose	3.83 ± 0.13 <sup>a</sup>	0.16 ± 0.02 <sup>b</sup>	0.85 ± 0.85 <sup>b</sup>	0.16 ± 0.01 <sup>b</sup>
Lactic acid	1.02 ± 0.02 <sup>c</sup>	3.92 ± 0.42 <sup>a</sup>	2.73 ± 0.03 <sup>bc</sup>	3.72 ± 0.34 <sup>ab</sup>
Acetic acid	0.97 ± 0.05 <sup>c</sup>	1.66 ± 0.07 <sup>ab</sup>	1.16 ± 0.13 <sup>bc</sup>	1.83 ± 0.20 <sup>a</sup>
Minerals (µg/mL)*				
Na	250 ± 10 <sup>b</sup>	280 ± 10 <sup>a</sup>	280 ± 10 <sup>a</sup>	280 ± 10 <sup>a</sup>
K	47 ± 35 <sup>b</sup>	745 ± 24 <sup>a</sup>	789 ± 18 <sup>a</sup>	738 ± 28 <sup>a</sup>
Ca	84 ± 1 <sup>c</sup>	200 ± 5 <sup>a</sup>	159 ± 3 <sup>b</sup>	214 ± 7 <sup>a</sup>
Mg	39 ± 1 <sup>b</sup>	30 ± 1 <sup>c</sup>	37 ± 1 <sup>b</sup>	49 ± 1 <sup>a</sup>
Fe	28 ± 1 <sup>b</sup>	31 ± 1 <sup>a</sup>	33 ± 1 <sup>a</sup>	31 ± 1 <sup>a</sup>
Cu	282 ± 2	264 ± 1	285 ± 6	264 ± 8
Mn	218 ± 3	224 ± 3	226 ± 3	215 ± 5
Free amino acids (µg/mL)**				
Aspartic acid	540	549	558	558
Threonine	70	89	97	90
Serine	46	11	61	10
Asparagine	49	50	61	49
Glutamic acid	251	264	265	261
Glycine	76	98	99	99
Alanine	610	642	655	649
Valine	101	151	142	154
Cystine	103	101	97	103
Methionine	47	52	53	52
Isoleucine	109	136	132	138
Leucine	402	433	437	439
Tyrocine	80	87	93	88
Phenylalanine	94	101	110	103
Tryptophan	Trace	Trace	Trace	Trace
Lysine	64	74	71	75
Histidine	14	16	17	16
Argine	156	185	ND	36
γ-aminobutyric acid	45	48	49	58
Ornithine	54	53	162	152
Total	2909	3140	3158	3131

\*Values are mean and SEM ( $n=3$ ). Values with different superscript with  $p < 0.05$

\*\*The triplicate samples shown were pooled and measured. Glutamine, proline, and taurine were not detected in all samples



value was not so low. The prebiotic effect of blue extract and pure phycocyanin in AFA on *Lactobacillus acidophilus* has been reported [37]. However, it is possible that glucose in AFA might maintain the growth of various LAB, similarly to this study.

### Mineral composition

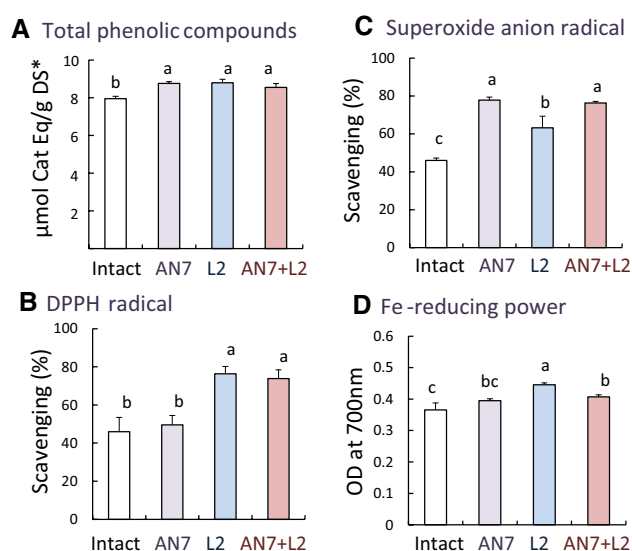
Soluble minerals in 10% AFA-S are also summarized in Table 1. Even in intact AFA-S, the concentration of K (0.48 mg/mL), Cu (0.28 mg/mL), and Mn (0.22 mg/mL) were high. Specifically, the ratio of K to Na (K/Na) was 1.9. During the fermentation, the Na concentration was only 1.1 times increased. On the other hand, K and Ca were approximately 1.6 times increased by the fermentation and the K/Na ratio was increased to approximately 2.7. Ca was increased 2.4 and 1.9 times with *L. plantarum* AN7 and *L. lactis* Kushiro-L2, respectively.

The K/Na ratio is important for people who take diuretics to control hypertension and who suffer from excessive excretion of potassium [38, 39]. Minerals, Ca, Cu, and Mn are also important as constituents of bones, teeth, and soft tissues and are vital for overall mental and physical wellbeing [40–42]. From the K and Ca concentrations that might be liberated due to acidification with lactic acid and acetic acid, the fermented AFA-S rather than the non-fermented AFA-S is promising.

### Free amino acids

AFA-S was rich in alanine (0.61 mg/mL), aspartic acid (0.54 mg/mL), and leucine (0.40 mg/mL), and these and total free amino acids tended to be high in the fermented AFA-S. Arginine was 0.16 and 0.18 mg/mL in intact 10% AFA-S and 10% AFA-S with *L. plantarum* AN7, respectively. It was not detected in 10% AFA-S fermented with *L. lactis* Kushiro-L2. It might be converted to ornithine.

The major free amino acids: alanine, aspartic acid, leucine, and glutamic acid, shown in this experiment are also major amino acids in various food materials. Besides taste, various food and medicinal functions of amino acids are known, e.g. liver protection, neural system improvement, and TCA cycle promoting compounds [43, 44]. Since the 1980s, arginine-ornithine exchange pathways in *Lactococcus lactis* have been known [45]. Various functions of both arginine and ornithine, such as improvement of gut function and liver protection, are also known [46, 47]. Furthermore, the antioxidant properties of alanine have been reported [48].



**Fig. 2** Total phenolic compound content (A), DPPH (B) and superoxide anion (C) radical scavenging capacities, and ferric reducing power (D) of 10% AFA-S fermented with *L. plantarum* AN7 and *Lactococcus lactis* Kushiro-L2. \*Catechin equivalents/g dry sample. **B–D**: 63 µL AFA-S/mL. Values are mean and SEM ( $n=3$ ). *a–c*: Values with different letters are significantly different at  $p < 0.05$

### Antioxidant properties of whole 10% AFA-S

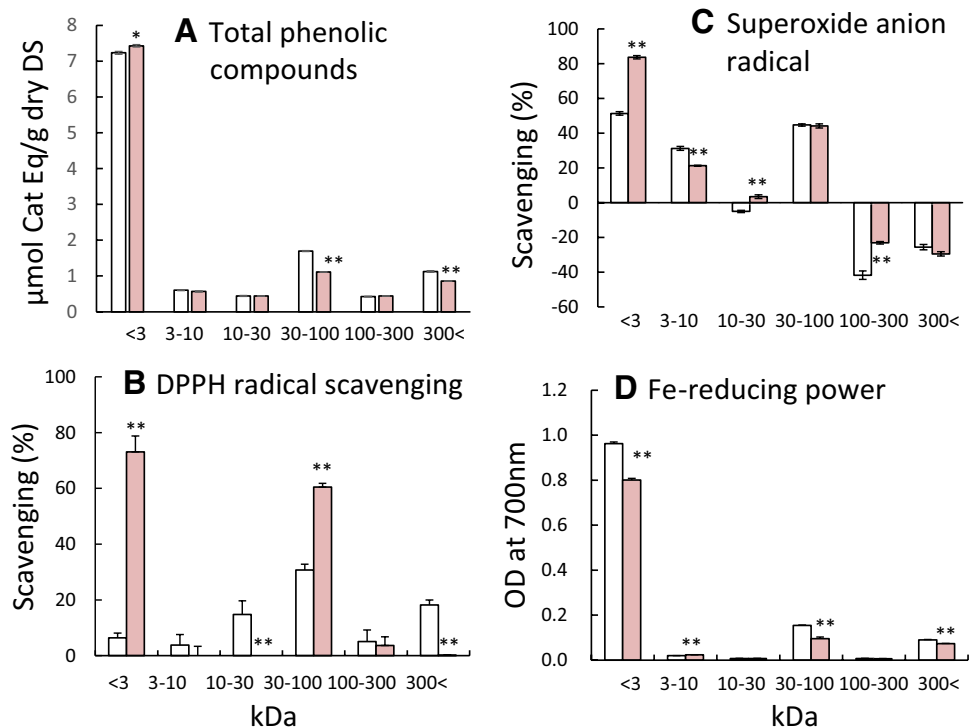
TPC in the AFA was 8 µmol CatEq/g dry samples and was little increased by the fermentation (Fig. 2A). It is closed to concentrations of the phenol group having amino acids phenylalanine and tyrosine content (Table 1).

In the antioxidant tests, the concentration of the intact sample that had 40–50% of max reaction value was used to determine the effect of the fermentation. DPPH radical scavenging capacity was increased by fermentation with *L. lactis* Kushiro-L2 but not with *L. plantarum* AN7 (Fig. 2B). The  $O_2^-$  scavenging capacity was high with *L. plantarum* AN7 compared with *L. lactis* Kushiro-L2 (Fig. 2C). The Fe-reducing power was slightly high with fermentation by *L. lactis* Kushiro-L2 (Fig. 2D). In all antioxidant assays, the 10% AFA-S fermented with the mixed culture of AN7 and Kushiro-L2 showed high capacities compared with the intact one. In the following experiments, intact and fermented mixed-cultures of 10% AFA-S were used.

### Antioxidant properties of the ultrafiltered fractions

Figure 3 shows the TPC and antioxidant properties of the ultrafiltered fractions. Most of TPC was smaller than 3 kDa, followed by 30–100 kDa and 300 kDa < compounds (Fig. 3A). During the fermentation, the low molecular TPC was little increased and the high molecular TPC was little decreased.

**Fig. 3** Total phenolic compound content (A), DPPH (B) and superoxide anion (C) radical scavenging capacities, and Fe-reducing power (D) in fractionated solutions of intact (open columns) or fermented (closed columns) AFA-S. Values are mean and SEM ( $n=3$ ). \* $p<0.05$ , \*\* $p<0.01$



Before the fermentation, the DPPH radical scavenging capacity was highest in 30–100 kDa, followed by 10–30 and 300 < kDa fractions (Fig. 3B). During the fermentation, the capacity was increased significantly in the < 3 kDa and 30–100 kDa fractions. The capacity of 10–30 kDa and 300 < kDa fractions disappeared with the fermentation.  $O_2^-$  radical scavenging compounds of intact AFA-S might be fractionated to < 3 kDa, 3–10 kDa and 30–100 kDa (Fig. 3C). In the case of 100 kDa and higher fractions,  $O_2^-$  radical or other NBT-oxidizable substances might be shown. The capacity in < 3 kDa, 10–30 kDa, and 100–300 kDa fractions were increased by the fermentation. The Fe-reducing power was high in the < 3 kDa and followed by 30–100 and 300 < kDa fractions (Fig. 3D). These were little decreased by the fermentation.

As mentioned in the introduction, the antioxidant properties of AFA with phycocyanin have been reported [23–27]. However, there are no reports about the activities of AFA fermented with LAB, though antioxidant, anti-inflammation, and anti-glycation capacities of some LAB-fermented edible algae had been reported [28–31].

From the results shown in Fig. 2, fermented AFA can be used more as an antioxidative food or supplement material. DPPH has been used extensively as a free radical to evaluate the reduction of substances in various foods including edible algae because it is simple and affordable [28, 31]. In previous reports about brown algae that containing phenolic compounds: phloroglucinols as antioxidant compounds show high DPPH radical scavenging capacity and

Fe-reducing power [28, 31, 33]. In these reports, TPC and the scavenging capacity were lowered by the fermentation. It might due to decomposition by the LAB. Therefore, the scavenging capacity of < 3 kDa and 30–100 kDa fractions shown in Fig. 3B were not correlated with the TPC. The isolation and identification of the active compounds in the fractions are needed for future studies.

In most organisms,  $O_2^-$  radicals are converted to hydrogen peroxide ( $H_2O_2$ ) by superoxide dismutase [49]. In the absence of transition metal ions, hydrogen peroxide is stable. However, hydroxyl radicals can be formed by the reaction of  $O_2^-$  with  $H_2O_2$  in the presence of metal ions, usually ferrous or copper [50]. Hydroxyl free radicals are much more reactive and toxic than  $O_2^-$ . Therefore, the  $O_2^-$  radical-scavenging activity shown in Fig. 2C suggests that the fermented 10% AFA-S can scavenge not only  $O_2^-$ , but also  $H_2O_2$  and hydroxyl radicals. This scavenging capacity (%) was well and moderately correlated with lactic acid and acetic acid concentrations shown in Table 1, approximately  $R^2 = 0.998$  and  $0.861$ , respectively. Furthermore, increasing the scavenging capacity in the < 3 kDa fraction shown in Fig. 3C may be due to mainly lactic acid. Lactate salts are known as antioxidative food additives [51]. Additionally, Groussard et al. [52] reported that lactate has  $O_2^-$  scavenging activity in both water and plasma. Further studies on NBT-oxidizable products in the high molecular fractions are needed.

Most non-enzymatic antioxidant activities, such as the scavenging of free radicals and the inhibition of

peroxidation, are mediated by redox reactions [53]. Compounds with reducing power are electron donors that can reduce the oxidized intermediates of lipid peroxidation processes and thereby act as primary and secondary antioxidants. The reducing power shown in Figs. 2D and 3D tended to be similar with the TPC result (Figs. 2A, 3A) and was slightly increased in < 3 kDa.

### Anti-glycation properties

BSA was used in this study to determine the anti-glycation property. Serum albumin can be glycosylated at multiple sites [12]. In the BSA-Fru model (Fig. 4A), anti-glycation activity of the 10% AFA suspension was increased clearly with the fermentation. The activity in fermented AFA was dose dependent manner. Before fermentation, the anti-glycation activity was mainly fractionated to < 3 kDa, 300 < kDa, and 30–100 kDa fractions (Fig. 4B). During the fermentation, the activity was increased only in 30–100 kDa fraction. On the other hand, it was decreased in 100 and higher kDa fractions. In the BSA-MGO model (Fig. 4C), the anti-glycation activity was shown with dose response manner and effect of the fermentation on the anti-glycation activity was small. The activity was mainly fractionated to < 3 kDa and 30–100 kDa (Fig. 4D).

Due to the AGE synthesis contain oxidation process, already there are reports about food materials have both antioxidant and anti-glycation activities with some phenolic compounds [15, 28, 29]. The anti-glycation activity of AFA suspension shown in BSA-MGO was correlated with

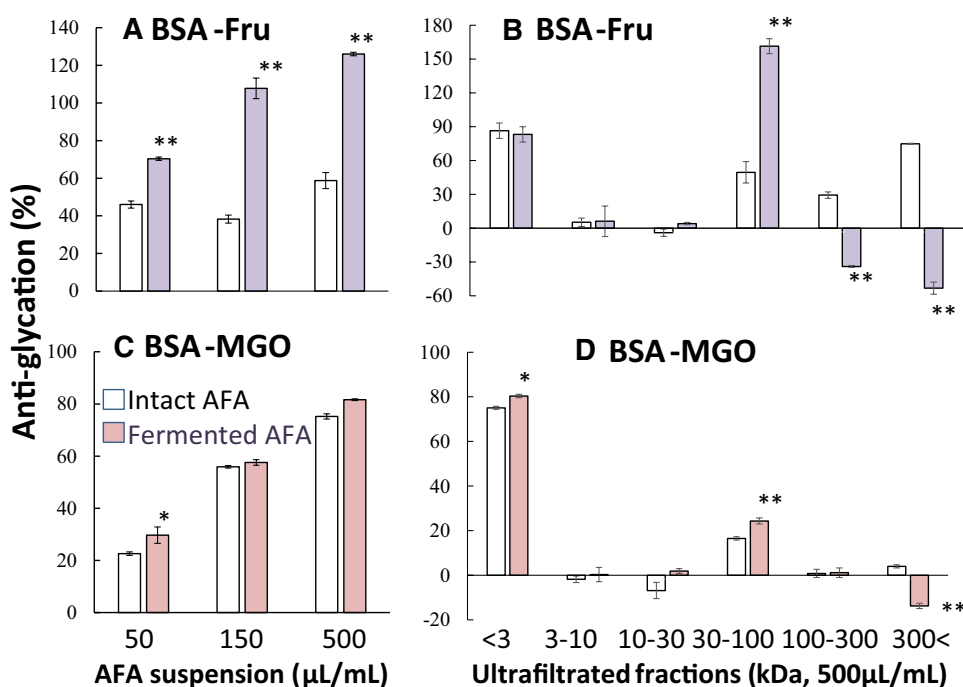
TPC (Fig. 3A,  $r^2 = 0.905$ ) and Fe-reducing power (Fig. 3D,  $r^2 = 0.933$ ). On the other hand, in the case of BSA-Fru model, although the correlation with any antioxidant capacities shown in Fig. 3 was not shown ( $r^2 < 0.489$ ), the activity increasing in 30–100 kDa fraction during the of the fermentation was agreed with DPPH radical scavenging capacity (Fig. 3B).

### Effects on NO secretion from murine macrophage RAW264.7 cells of whole 10% AFA-S

Without LPS and intact 10% AFA-S (Fig. 5A), the NO secretion in the culture media was increased with a concentration response at 2.0  $\mu\text{L}/\text{mL}$  and less sample. The NO secretion at 1.0–3.9  $\mu\text{L}/\text{mL}$  samples was increased by the fermentation. In the higher sample concentration, the induction was reverse concentration dependent. To confirm the reverse dependence, the cell toxicity of the high sample concentration was determined using WST-8 (Fig. 5B). Only 125  $\mu\text{L}/\text{mL}$  sample of intact 10% AFA-S lowered 27% in viability, though this cell toxicity was not observed in the fermented 10% AFA-S. The LPS induced NO was decreased by 16  $\mu\text{L}/\text{mL}$  and higher samples (Fig. 5C). The inhibition activity of the 10% AFA-S was not affected by the fermentation.

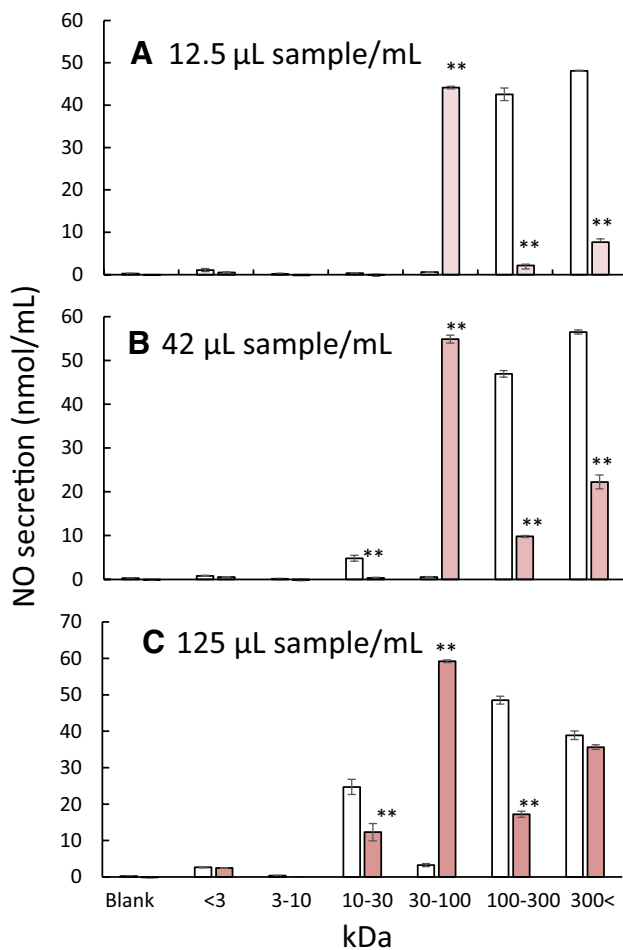
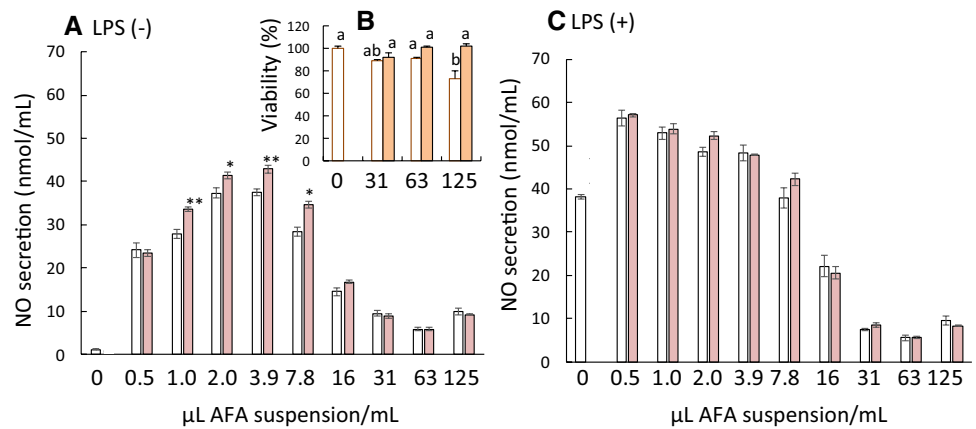
To determine the existence of fermented products with an induction capacity to NO secretion, the NO with the ultrafiltered fractions without LPS was determined. The NO secretion without LPS was induced clearly by 100 kDa and higher molecular fractions (Fig. 6). The induction was not high in the high sample concentration

**Fig. 4** Effect of intact AFA-S (open columns) or AFA-S fermented with *L. plantarum* AN7 and *L. lactis* L2 (closed columns) on anti-glycation activity shown in BSA-fructose (A, B) and BSA-methylglyoxal models (C, D). Values are mean and SEM ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$





**Fig. 5** Effect of intact AFA-S (open columns) or AFA-S fermented with *L. plantarum* AN7 and *L. lactis* L2 (closed columns) on NO secretion from murine macrophage RAW264.7 cells without (A) or with (C) LPS. (B) Cell viability in the high concentration samples confirmed by WST-8. Values are mean and SEM ( $n=3$ ). \* $p<0.05$ , \*\* $p<0.01$ . Values in (B) with different superscript letters are significantly different at  $p<0.05$



**Fig. 6** NO secretion from murine macrophage RAW264.7 cells induced by low (A), moderate (B), and high (C) concentrations of molecular size fractions of non-fermented AFA solution (open columns) or fermented AFA solution (closed columns). Values are mean and SEM ( $n=3$ ). \* $p<0.05$ , \*\* $p<0.01$

(Fig. 6C); rather than the less concentration (Fig. 6A, B). This induction activity of the high molecular fractions was decreased by the fermentation. Furthermore, the high molecular fractions of fermented AFA-S dependent on the concentration. After the fermentation, the induction activity in 30–100 kDa fraction was very high. It can be considered that immunomodulation activity induced by the fermentation was correlated with DPPH radical scavenging (Fig. 3B) and anti-glycation, in BSA-Fru model (Fig. 3B), activities.

The immunomodulation activity of extracted compounds from AFA, such as polysaccharides, chromoprotein-related compounds (phycobilins and phycobiliproteins), and low molecular (<5 kDa) fraction, had been reported [54–56]. As shown in Fig. 5, AFA might have immune-promoting activity in lower concentrations and anti-inflammatory activity in high concentrations. Furthermore, the immune-modulating activity provided as NO secretion without LPS was increased by the fermentation (Fig. 5A). These phenomena were also observed for a green loofah *Luffa cylindrica* suspension [20]. Because some selected LAB strains have immune-modulating activity [19, 57], it is thought that the increasing NO secretion was the result of additives or the synergistic effect of AFA and LAB cells. However, as shown in Fig. 6, the immunomodulation activity of 100 kDa and higher molecular fractions was decreased by the fermentation and it was increased significantly in the 30–100 kDa fraction. This result suggests that the active compounds in fermented AFA-S are different from high molecular compounds including cell membranes and <3 kDa compounds, already reported and mentioned above. In this fraction, the DPPH radical scavenging capacity was increased by the fermentation (Fig. 3B). The isolation and identification of the active compounds in the 30–100 kDa fraction, including phycobiliprotein-related compounds, are also needed.

The results of this study suggest that the fermented AFA-S can play antioxidant, anti-glycation and immunomodulation

roles in food and supplement materials. These activities are higher than in the non-fermented AFA-S. Future studies regarding the isolation of metabolites generated during fermentation and their biological effects on cultured macrophages and *in vivo* are needed.

## Conclusion

To clarify the effect of LAB fermentation on the antioxidant and immunomodulatory capacities of AFA, hot aqueous extract suspensions made from dry-powdered AFA (10% AFA-S) were fermented by *L. plantarum* AN7 and *L. lactis* Kushiro-L2 strains isolated from a coastal region in Japan. Then, the *in vitro* antioxidant properties and immunomodulation effects on NO secretion from RAW264.7 cells of the non-fermented and fermented 10% AFA-S were determined. The DPPH and O<sub>2</sub><sup>-</sup> radical scavenging capacities and Fe-reducing power were increased in the mixed culture of AN7 and Kushiro-L2. The DPPH radical scavenging capacity of the fermented AFA-S was fractionated to mainly < 3 kDa and 30–100 kDa. The O<sub>2</sub><sup>-</sup> radical scavenging capacities of the fermented AFA-S were fractionated to mainly < 3 kDa. Anti-glycation activity in BSA-fructose model rather than BSA-methylglyoxal model was increased by the fermentation. The increased anti-glycation activity was fractionated to mainly 30–100 kDa. Without LPS, 2.0 μL/mL and less non-fermented 10% AFA-S increased the NO secretion in the murine macrophage RAW264.7 culture media with a concentration response. The NO secretion activity was increased by the fermentation. The increased immunomodulation capacity in the fermented AFA-S was also fractionated to mainly 30–100 kDa. These results suggest that fermented AFA-S is a more useful material for health foods and supplements. Further study on the active compounds in < 3 kDa and 30–100 kDa fractions are needed in the future.

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## Compliance with ethical standards

**Conflict of interest** The authors do not declare any conflicts of interest.

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