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ORIGINAL ARTICLE

Effects of fermented green-loofah and green-papaya on nitric oxide secretion from murine macrophage raw 264.7 cells

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Abstract

To clarify the effect of lactic acid bacteria (LAB) fermentation on the immunomodulation capacity of green-loofah and green-papaya, aqueous suspensions prepared from the fresh and dry-powdered vegetables were fermented by *Lactococcus lactis* subsp. *lactis* Uruma-SU1 and *Lactobacillus plantarum* Uruma-SU4. Fermented and non-fermented suspensions were added to murine macrophage RAW264.7 culture with and without *Escherichia coli* O111 lipopolysaccharide (LPS). In the absence of LPS, nitric oxide (NO) secretion was elevated significantly in LAB fermented suspensions compared to that in non-fermented suspensions. NO production in fermented suspensions was observed even at low sample concentrations, but it was attenuated in the centrifuged supernatant. With LPS treatment, inhibition of NO secretion was shown with the high concentration of the non-fermented and also fermented samples. These results suggest that fermented green-loofah and green-papaya suspensions can play both immunostimulatory and anti-inflammatory roles at low and high doses, respectively.

Keywords Green-loofah · Green-papaya · *Lactobacillus plantarum* · Murine macrophage RAW264.7 · Nitric oxide

Introduction

Reactive oxygen species (ROS), such as superoxide anion (O_2^-) radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen, are generated in the bodies of aerobic organisms [1]. ROS react with important cellular components, such as DNA, proteins, lipids, and other small cellular molecules and induce a wide range of common diseases and age-related degenerative conditions, such as cardiovascular disease, inflammation, and cancer; as well as neurodegenerative diseases, including Alzheimer's disease [2–5]. 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 [6]. NO directly modifies its intracellular targets due to its ability to passively permeate the cellular membrane, and it plays important roles in inflammation and age-related diseases as mentioned in above [7].

Anti-inflammatory effects of various vegetables, fruits and ethnic medicinal plants evaluated by the inhibitory effect on NO secretion from macrophages have been reported. For example, *Ligularia fischeri* (a Korean vegetable), red raspberries, and *Polygonum multiflorum* (a traditional Chinese herb), have all shown inhibitory effects on *Escherichia coli* lipopolysaccharide (LPS)-induced NO production in murine RAW264.7 macrophages [8–10]. 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 [11–14].

Ripened brown-loofah (*Luffa cylindrica*), and yellow-papaya (*Carica papaya*) are widely known as sponge materials and sweet fruits, respectively. On the other hand, green-loofah and green-papaya have also been used as traditional vegetables in warm areas, such as India, Africa, and Latin America. The Ryukyu Islands, belonging to the Okinawa Prefecture, Japan, are located in the subtropical zone (24.0–27.0N; 122.9–131.3E), which is suitable for the cultivation of these vegetables. Usually, harvested vegetables that do not have a good physical appearance are either discarded or a part of the produce is used as a powder and puree for health food products. When the balance between supply,

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demand, and distribution breaks down, many of these vegetable products are sometimes discarded. Furthermore, fruits and vegetables have the highest wastage rates at retail and consumer levels [15].

The antioxidant properties of both loofah and papaya have been reported [16, 17]. The inhibitory effect of loofah on LPS-induced NO production from RAW264.7 cells has also been reported [18]. Some LABs isolated from coastal regions can increase the anti-oxidant (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 plants during fermentation [19–22]. In the present study, we sought to clarify the effect of LAB fermentation on the immunomodulatory capacity of green-loofah and green-papaya. Hot aqueous extract suspensions made from the fresh and dry-powdered vegetables were fermented by *Lactococcus lactis* subsp. *lactis* Uruma-SU1 and *Lactobacillus plantarum* Uruma-SU4; then, effects of the non-fermented and fermented suspensions on NO secretion from RAW264.7 cells were observed with and without LPS treatment.

Materials and methods

Bacterial strains

In a previous study [23], *L. lactis* subsp. *lactis* Uruma-SU1 (Accession No. LC209104) and *L. plantarum* Uruma-SU4 (Accession No. LC213630) were isolated from algal beach casts on the coast of Okinawa Island and wild grasses in Miyakojima Island, respectively, and stocked with Microbank beads (Iwaki Co., Tokyo, Japan) at − 80 °C. To prepare for experimentation, these strains were inoculated into 3 mL of de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. The pre-incubated culture was centrifuged at 3000×*g* at 4 °C for 5 min, and the pelleted cells were suspended in distilled water (DW).

Preparation of aqueous suspensions from fresh (ASF) and dried (ASD) vegetables

Fresh green-loofah *L. cylindrica*, and green-papaya *C. papaya* (Fig. 1A, B) were purchased from a retail farmer's shop in Okinawa and Miyakojima Islands, respectively. A

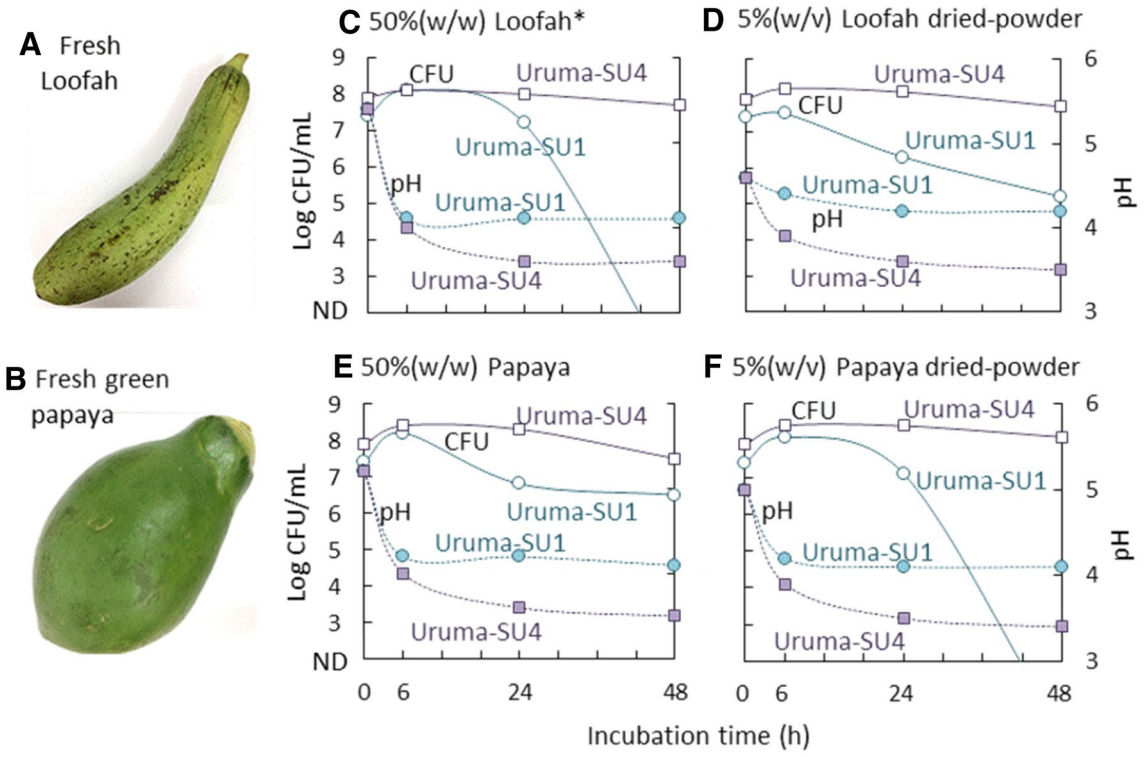

Fig. 1 Images of green-loofah and green-papaya (**A**, **B**), and changes in the viable counts and pH in green-loofah (**C**, **D**) and green-papaya (**E**, **F**) Suspensions inoculated *Lactobacillus lactis* subsp. *lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4 and incubated at 37 °C. **C**, **E** *Fresh vegetables were autoclaved with the same weight of DW. **D**, **F** Dried and powdered vegetables were autoclaved with 20× weight of DW. Values are mean and standard error of mean (SEM, $n = 3$)

part of the fresh green-loofah was sun-dried, milled using a blender (Oster 16 Speed Blender; Osaka Chemical Co., Japan) and sieved through a 1 mm^2 mesh. Freeze-dried powder of green-papaya was purchased from Kimusshi-factory (Miyakojima-city, Okinawa, Japan). According to Food Composition Database of Ministry of Education, Culture, Sports, Science and Technology-Japan (MEXT, http://www.mext.go.jp/component/a_menu/science/detail/__icsFiles/afieldfile/2017/12/25/1374049_1r12.xlsx) water content of green-loofah and green-papaya are 95 and 89% (w/w), respectively. The fresh vegetables (100 g each) were homogenised with 100 mL DW using the blender. The dried powders (10 g each) were suspended in 190 mL of DW. These suspensions were autoclaved (121 °C for 15 min) and used as aqueous suspensions of fresh green-loofah and green-papaya and their dried vegetables.

Fermentation of the vegetable suspensions by *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4

The prepared LAB suspension in DW (0.3 mL) was inoculated into 30 mL of the prepared vegetable suspension, and incubated at 37 °C for 48 h. To evaluate the fermentation, the pH value was measured using a pH meter (LAQUA Twin B-711, Horiba) at 0, 6, 24 and 48 h during incubation. At the same time, viable plate count was measured using MRS agar plate [24]. Following incubation, the sample suspension was centrifuged at 3000×*g* at 4 °C for 5 min and the supernatant was filtered using a 0.2 µm filter to analyse saccharides and lactic acid by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: column, ICSep 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.

Effects on NO secretion by RAW264.7 murine macrophages

The sample suspensions before and after fermentation were adjusted to a pH of 8.0 by using 1 mol/L NaOH and then heated in boiling water for 20 min. A half-volume of the sterilised suspension was centrifuged at 3000×*g* at 4 °C for 5 min, and the supernatant was collected. The suspension and supernatant were mixed with 1.5 volumes of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 5% v/v foetal bovine serum (FBS), and were serial diluted with same media.

To determine the immune stimulation property, murine macrophage like RAW264.7 cells (TIB-71; American Type Culture Collection, Manassas, VA) were used [25]. 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 °C for 2 h in an atmosphere of 5% CO_2, the medium was replaced with fresh medium (0.1 mL), and the medium (0.1 mL) that containing or not containing (control) sample was added. After a 20 h incubation, the NO concentration in the cultured medium was determined with 10% (w/v) Griess-Romijn nitrite reagent (Wako Pure Chemical, Osaka, Japan) as described previously [26].

For the anti-inflammation assay, LPS from *E. coli* O111 B4 (Sigma-Aldrich, St. Louis, MO) 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.

Statistical analysis

The measured values ($n=3$) are presented as means ± standard error of the mean (SEM). In the case of the data about NO secretion, one-way ANOVA was performed to assess differences among groups, and individual means were compared by Tukey's post hoc test, using statistical software (Excel Statistic Ver. 6, Esumi, Tokyo, Japan). Significant differences were accepted at $p<0.05$.

Results and discussion

Fermentation Properties of *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4 in green-loofah and green-papaya Suspensions

Changes of LAB viable counts and pH values are shown in Fig. 1C–F. The pH values of the suspensions made from fresh green-loofah and green-papaya were 5.5 and 5.3, respectively. The values of sun-dried green-loofah and freeze-dried green-papaya were 4.6 and 5.0, respectively. It is possible that the sample vegetables might be acidified by the drying processes. In all of the sample suspensions, we observed a lower pH value with *L. plantarum* Uruma-SU4 (approximately 3.5) compared to *L. lactis* Uruma-SU1 (approximately 4.1). After 6 and 24 h incubation with Uruma-SU1 and Uruma-SU4, respectively, pH did not change significantly. The viable count of Uruma-SU4 was kept at a level 7.5 and higher log CFU/mL. However, the counts of Uruma-SU1 increased during the first 6 h of incubation, and then decreased, notably, in suspensions made from fresh green-loofah and freeze-dried

Table 1 Saccharides and lactic acid contetnts in ageous extract suspensions (AES) of loofah and papaya fermented with *L. lactis* Uruma-SU1 or *L. plantarum* Uruma-SU4 (mg/mL)

	Intact	Fermented with	
		L. lactis Uruma-SU1	*L. plantarum* Uruma-SU4
AES of 50% fresh loofah			
Glucose	16.61 ± 0.06	13.23 ± 0.16	7.11 ± 0.19
Fructose	7.41 ± 0.03	6.86 ± 0.10	6.61 ± 0.04
Lactic acid	–	2.97 ± 0.06	7.05 ± 0.02
AES of 5% dried loofah			
Glucose	15.93 ± 0.30	15.97 ± 0.30	5.69 ± 0.59
Fructose	9.62 ± 0.23	9.70 ± 0.56	8.91 ± 0.86
Lactic acid	–	1.55 ± 0.09	6.88 ± 0.39
AES of 50% fresh papaya			
Sucrose	0.43 ± 0.01	0.47 ± 0.01	Trace
Glucose	15.60 ± 0.13	11.93 ± 0.36	6.34 ± 0.30
Fructose	6.65 ± 0.05	5.56 ± 0.12	Trace
Lactic acid	–	2.48 ± 0.10	9.19 ± 0.13
AES of 5% dried papaya			
Sucrose	0.15 ± 0.05	0.99 ± 0.03	1.69 ± 0.09
Glucose	16.02 ± 0.16	15.32 ± 0.15	5.93 ± 0.21
Fructose	10.52 ± 0.12	11.66 ± 0.10	6.90 ± 0.23
Lactic acid	–	2.57 ± 0.02	11.01 ± 0.28

Values are mean and SEM ($n = 3$); – not detected

green-papaya (Fig. 1C, F), Uruma-SU1 could not be detected (< 2 log CFU/mL) after 48 h fermentation.

Saccharide and lactic acid concentrations in the vegetable suspensions before and after the fermentation were summarised in Table 1. Prior to fermentation, there were no large differences in the saccharide content among the sample suspensions. For instance, glucose, fructose, and sucrose concentrations were 15.6–16.6, 6.7–10.5 mg/mL, and less than 0.4 mg/mL, respectively. After incubation, 1.6–3.0 and 6.9–11.0 mg/mL of lactic acid were generated with Uruma-SU1 and Uruma-SU4, respectively. The concentration of lactic acid with Uruma-SU4 was higher in the green-papaya samples than the green-loofah samples.

These results suggest that Uruma-SU4 is more suitable for fermentation of green-loofah and green-papaya, though both LAB strains are capable of fermenting these products. In general, the final pH of broth and biomaterials cultured with *L. lactis* is at least 4.3, whereas the value with *L. plantarum* was approximately 3.5 [22, 27]. On the other hand, there are many reports about bacteriocins produced by *L. lactis* that inhibit the growth of various Gram-positive bacteria [28]. In our preliminary study, Uruma-SU1 suppressed the growth of the Gram-positive *Listeria monocytogenes, L. plantarum* and other *L lactis* strains. The antibacterial compound along with lactic acid may have suppressed growth of Uruma-SU1 itself.

Effects of fermented green-loofah suspension and supernatant on NO secretion from RAW264.7 cells

In the absence of LPS, non-fermented suspensions and supernatants made from fresh green-loofah stimulate very low levels of NO secretion (Fig. 2A, B). Fermentation of the suspension increased NO production significantly; however, the stimulation activity was negatively correlated with concentration. An increase in NO production by fermentation was not observed in the supernatant.

In the cultured medium, NO concentration was increased by treatment with *E. coli* LPS (Fig. 2C, D). The inhibitory effect on LPS-induced NO secretion is regarded as anti-inflammatory activity. The non-fermented green-loofah suspension and supernatant showed a dose-responsive inhibitory effect. The concentration of NO in cultured medium with fermented green-loofah, particularly in the suspensions, was higher than that of the cultured medium with nonfermented green-loofah.

Without LPS treatment, NO production was also very low in non-fermented suspensions made from sun-dried green-loofah and was greatly increased in fermented samples (Fig. 4A). This immunostimulatory activity associated with fermentation was also clearly shown in the supernatant (Fig. 4B). With the addition of LPS, similar anti-inflammatory effects were shown to those of the samples made from fresh green-loofah (Fig. 3C, D).

Although the anti-inflammatory property of green-loofah, expressed as an inhibitory effect on NO production in RAW264.7 cells, has been reported [18], the immunostimulatory effect has not been clarified. From our results demonstrating low NO activity in supernatants made from fresh green-loofah (Fig. 2B), we suggest that the immunostimulatory capacity of fermented green-loofah is correlated with the activity of LAB cells. Indeed, induction of NO from RAW264.7 cells with LAB, including *L. plantarum* and *L. lactis*, has been shown in previous studies [26, 29, 30]. Alternatively, the NO induction by suspensions of sun-dried green-loofah without fermentation may be attributed to compounds generated during the drying process. Further studies about the generation of active compounds are needed in future.

Effects of fermented green-papaya suspension and supernatant on NO secretion by RAW264.7 cells

Without LPS treatment, suspensions and supernatants made from non-fermented fresh green-papaya induced very low levels of NO secretion (Fig. 4A, B). The induction of high levels of NO by fermentation and the decrease in NO production in the supernatant were similar with the results observed in the green-loofah sample. Furthermore, as in the

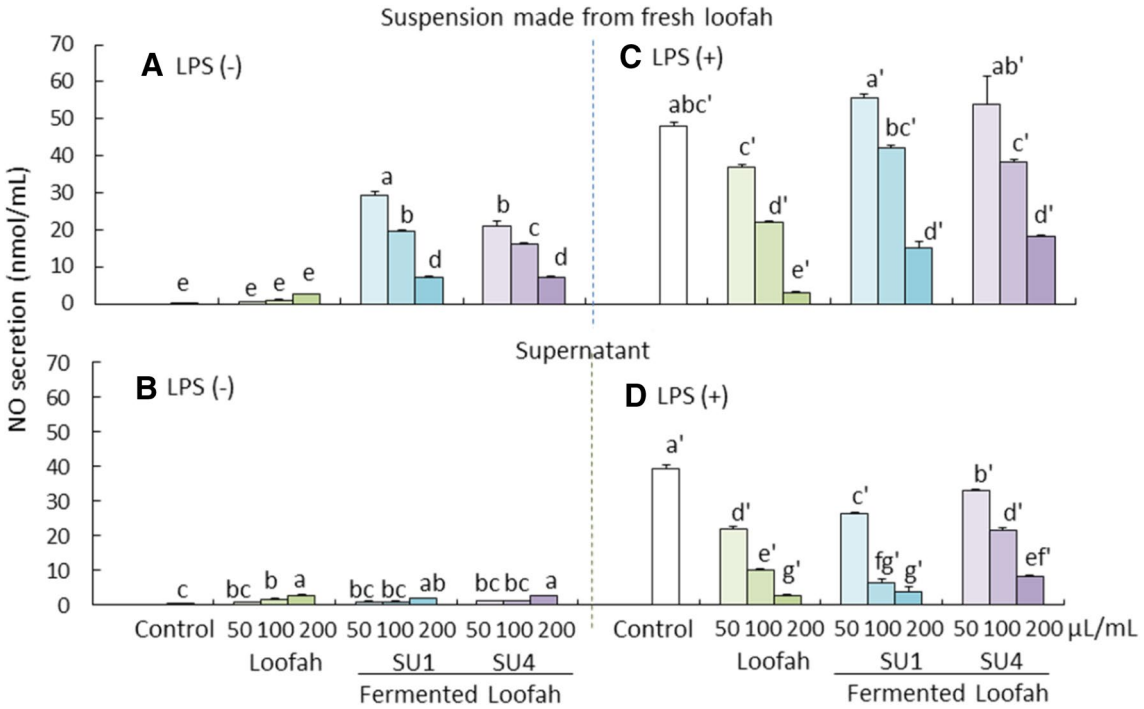

Fig. 2 Nitric oxide secretion from RAW264.7 cells cultured with suspension (**A**, **C**) and supernatant (**B**, **D**) made from fresh green-loofah, fermented by *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4. Samples were cultured without (**A**, **B**) or with (**C**, **D**) *E. coli* O111 LPS. Values are mean and SEM ($n=3$). Values with different superscript letters are significantly different ($p<0.05$)

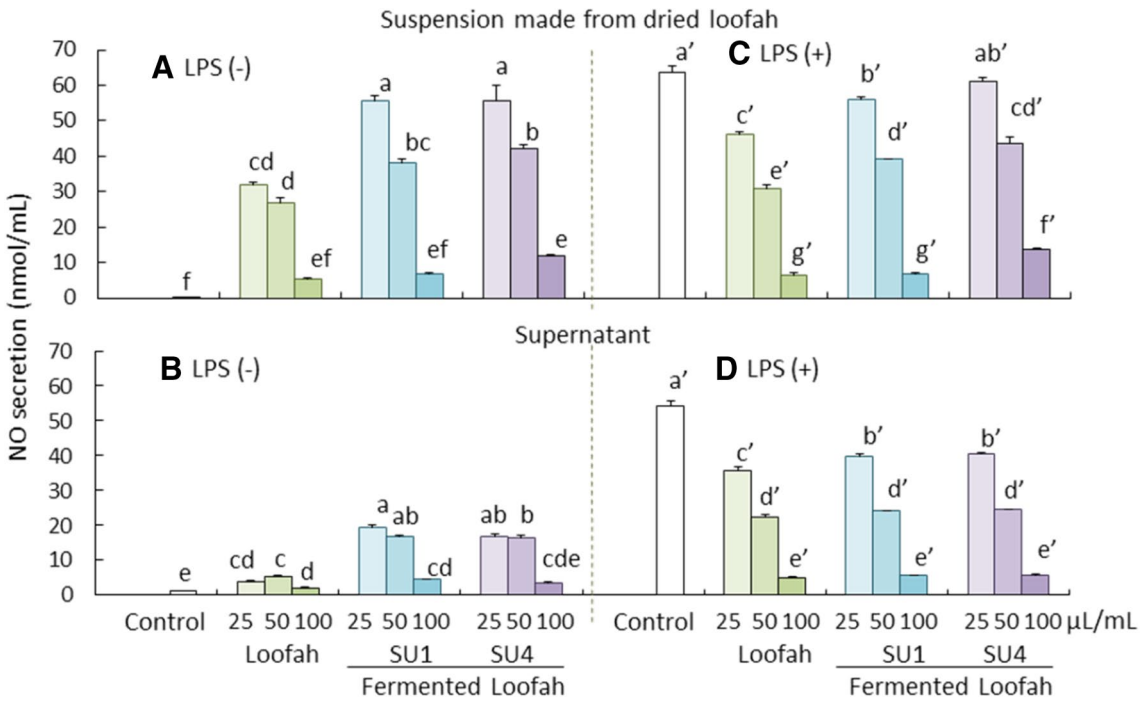

Fig. 3 NO secretion from RAW264.7 cells cultured with suspension (**A**, **C**) and supernatant (**B**, **D**) made from sun-dried green-loofah, fermented by *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4. Samples were cultured without (**A**, **B**) or with (**C**, **D**) *E. coli* O111 LPS. Values are mean and SEM ($n=3$). Values with different superscript letters are significantly different ($p<0.05$)

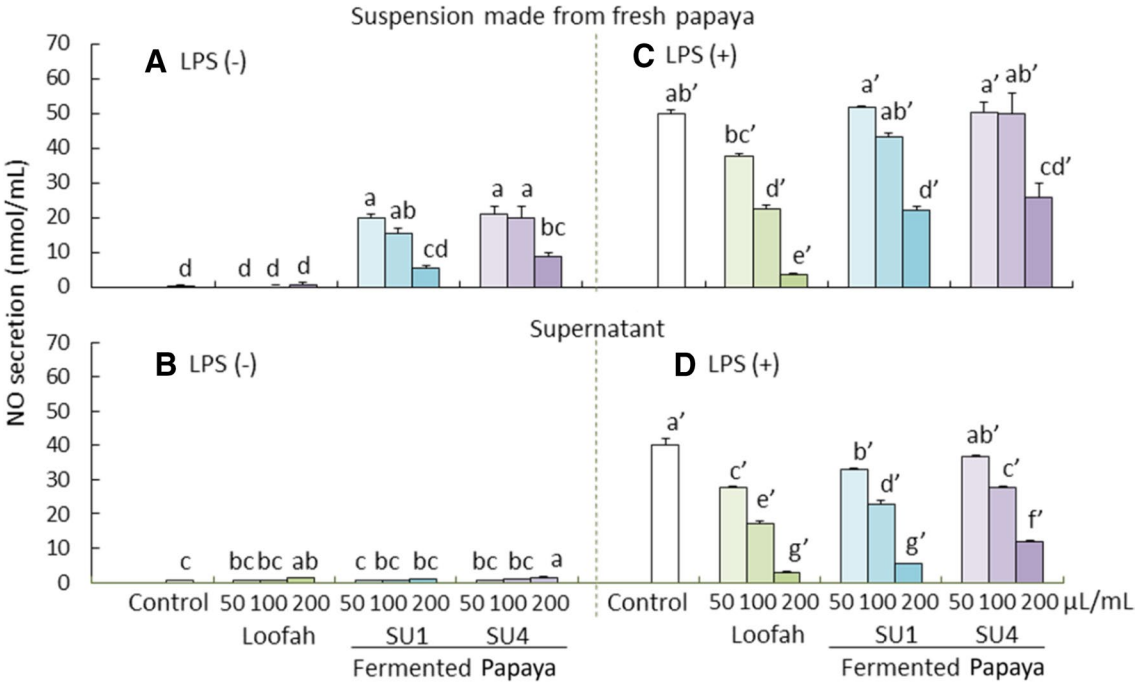

Fig. 4 NO secretion from RAW264.7 cells cultured with suspension (**A**, **C**) and supernatant (**B**, **D**) made from fresh green-papaya, fermented by *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4. Samples were cultured without (**A**, **B**) or with (**C**, **D**) *E. coli* O111 LPS. Values are mean and SEM ($n=3$). Values with different superscript letters are significantly different ($p<0.05$)

green-loofah samples, we observed no increase in NO production in the LPS-treated samples following fermentation (Fig. 4C, D).

In the absence of LPS, NO production in samples made from freeze-dried green-papaya was very low (Fig. 5A, B). Fermentation with *L. lactis* Uruma-SU1 did not significantly increase the NO activity in either suspensions or supernatants (Fig. 5A). With LPS treatment, the anti-inflammatory effects observed were similar to those of the other samples, although this activity tended to be stronger in these samples (Fig. 5C, D).

Though the inhibitory effect of some of the extract solutions and chemical compounds from the leaves of green-papaya on LPS-induced NO secretion in RAW264.7 cells has been previously reported [31], the inhibitory effect of green-papaya fruit has not been elucidated.

These results suggest that water extract suspensions of green-loofah and green-papaya without LAB fermentation have an anti-inflammatory effect. Our results further suggest that the fermented vegetable suspensions have both immunostimulatory and anti-inflammatory capacities. However, the immunostimulatory capacity observed was negatively correlated with the concentration of the suspension. It can be considered that the compounds showing anti-inflammatory capacity in the high dose samples may suppress the production of in RAW264.7 stimulated by the LAB cells. Therefore, we evaluated the NO induction at a lower concentration of the samples in the absence of LPS in the next experiment.

NO secretion by low dose samples without LPS treatment

Figure 6 shows NO secretion by low dose samples remade from sun-dried green-loofah without LPS treatment. NO secretion peaked in samples with concentrations of 3.1 and 6.3 µL/mL. In the case of sample concentrations 0.39–6.3 µL/mL, the NO secretion was increased by both Uruma-SU1 and Uruma-SU4. In this experiment, the NO secretion by the samples was tended to be lower than ones of former experiment (Fig. 3A). It is considered that harvest date and freshness of vegetables affected fermentation process and the functionality.

In previous studies, O_2^- radical scavenging activity in aqueous extracts from edible plants was shown to be induced by fermentation with *L. plantarum* [21, 26, 32]. In some of these reports, the fermented samples showed protective and anti-inflammatory effects on cultured enterocyte-like cells, macrophages, and dextran sodium sulphate (DSS)-induced intestinal bowel disease (IBD) in mice as well as immunomodulation properties in RAW264.7 cells. It has been proposed that the correlation and balance of O_2^- and

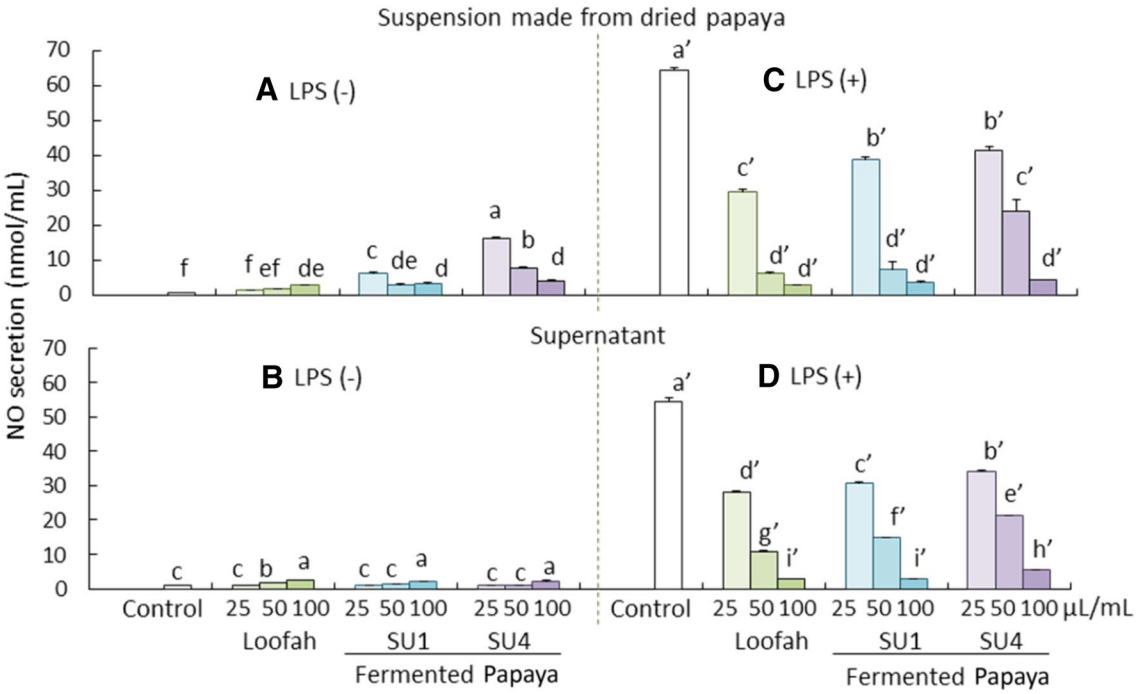

Fig. 5 NO secretion from RAW264.7 cells cultured with suspension (**A**, **C**) and supernatant (**B**, **D**) made from freeze-dried green-papaya, fermented by *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4. Samples were cultured without (**A**, **B**) or with (**C**, **D**) *E. coli* O111 LPS. Values are mean and SEM ($n=3$). Values with different superscript letters are significantly different ($p<0.05$)

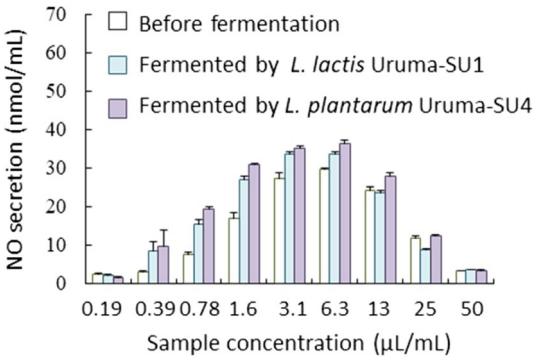

Fig. 6 NO secretion from RAW264.7 cells cultured with low concentration suspensions made from sun-dried green-loofah non-fermented (open columns), and fermented by *L. lactis* Uruma-SU1 (semi-closed columns) and *L. plantarum* Uruma-SU4 (closed columns). Samples were cultured without LPS. Values are mean and SEM ($n=3$)

NO is important for the prevention of ROS-related diseases [33–35].

The results of our study suggest that the fermented green-loofah and green-papaya suspensions can play both of immunostimulatory and anti-inflammatory roles at low and high doses, respectively. Due to the different results observed between the suspension and the supernatant, the active compounds in LAB cells must also be considered. However, differences in effect on NO production between the LAB strains varied by each sample. Thus, future studies regarding the metabolites generated during fermentation and their biological effects on cultured macrophages and in vivo are needed.

Conclusion

In present study, we sought to clarify the beneficial properties of green-loofah and green-papaya fermented with *L. lactis* Uruma-SU1 and *L. plantarum* Uruma-SU4. The immunostimulatory and anti-inflammatory properties of these fermented samples were compared with ones of the non-fermented samples in murine macrophage RAW264.7 cells. In the absence of LPS, NO production was low in non-fermented green-loofah and green-papaya and it was significantly elevated in fermented samples. This immunostimulatory activity was shown even at low sample concentrations and it was eliminated by the centrifugation of the samples. LPS-induced NO secretion was suppressed by high concentrations of non-fermented suspensions and supernatants. This anti-inflammatory activity was not induced by fermented samples. These results suggest that fermented green-loofah and green-papaya suspensions exert both

immunostimulatory and anti-inflammatory functions at low and high doses, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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