

Effects of Holdfast of *Laminaria japonica* on *Listeria* Invasion on Enterocyte-Like Caco-2 Cells and NO Production of Macrophage RAW 264.7 Cells

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Received: 15 May 2012 / Accepted: 6 August 2012 /
Published online: 15 August 2012
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Abstract *Listeria monocytogenes* (Lm) causes food poisoning in humans mainly through consumption of ready-to-eat foods. Immunocompromised persons are at the highest risk for infection. We investigated effects of crude soluble polysaccharides (SPS) and ethanolic extract (EE) fractions of frond (kombu) and holdfast (ganiashi) parts of *Laminaria japonica* on Lm invasion into human enterocyte-like Caco-2 cells and immune and/or inflammatory reactions of murine macrophage RAW 264.7 cells. Recovery and viscosity were high in kombu SPS. Total phenolic content and antioxidant activities (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity and Fe-reducing power) were higher in ganiashi EE. EE of ganiashi, rather than kombu, suppressed the Lm invasion into the differentiated Caco-2 cells, though the inhibitory effect of SPS was not significant. Ganiashi SPS increased the nitric oxide (NO) production of intact RAW 264.7 cells. On the other hand, the NO production from *Escherichia coli* O111 lipopolysaccharide-activated cells was suppressed by kombu SPS and ganiashi EE. These results suggest that *L. japonica*, particularly ganiashi, might suppress the invasion and infection of Lm and also the inflammation.

Keywords *Laminaria japonica* · Holdfast · *Listeria monocytogenes* · Caco-2 · RAW 264.7

Introduction

Listeria monocytogenes (Lm) causes listeriosis in humans mainly through consumption of ready-to-eat foods [1]. Immunocompromised persons, the elderly, and pregnant women and their fetuses or newborns are at the highest risk for infection [2]. In Europe and North America, Lm has been observed in various foods, including salted and/or fermented protein-rich foods

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such as cheese and smoked salmon [3, 4] and has occasionally been the causative agent in outbreaks [5].

In East Asian countries, including China, Korea, and Japan, many kinds of edible algae, particularly various brown algae, are harvested as edible plants [6]. Among dried algal products, the supply of kombu (dried frond of *Laminaria japonica*, the largest edible brown algae) is more abundant than other popular edible algae, such as wakame *Undaria pinatifida* and nori *Porphyra* spp. products [7]. Furthermore, kombu has been a popular seasoning in Japanese cuisine since ancient times; dried kombu products are rich in glutamate salt, which is the most well-known umami-tasting compound [8]. Brown algae are also known to have notable bioactive compounds such as water-soluble polysaccharides (SPS), including alginic acid, fucoidan and laminaran, and phlorotannins called algal polyphenols [9]. Fucoidan and phlorotannins are regarded as immune-improving compounds correlated with antiviral, antibacterial, and antioxidative activities [10, 11]. On the other hand, alginate and laminaran are fermented by intestinal microbiota and exert host effects similar to prebiotics [12, 13]. The ameliorating effect of some prebiotics, such as oligofructose, against Lm infection has been reported [14].

The holdfast part of *L. japonica* is called ganiashi by the local fisheries. It is attached firmly on the kombu cultivation net. Although ganiashi has traditionally been regarded as waste, recently, some food and medicinal functions of ganiashi have been reported, such as cytotoxic activity against human breast cancer cells and adenocarcinoma tumors in mice [15, 16]. Interestingly, the fucoidan present in ganiashi is rich in uronic acid and differs in saccharide composition from that in the frond.

Nitric oxide (NO) is a highly reactive free radical involved in a number of physiological and pathological processes [17]. NO may play an important role in the pathophysiology of various diseases and its role in macrophage toxicity is well-established [18]. Furthermore, NO acts as a cytotoxic agent in the immunological interaction between invading microorganisms and macrophages [19]. In this study, we investigated effects of crude SPS and ethanolic extract (EE) fractions of the frond (kombu) and holdfast (ganiashi) parts on the Lm invasion into human enterocyte-like Caco-2 cells and immune and/or inflammatory reactions of murine macrophage RAW 264.7 cells.

Materials and Methods

L. monocytogenes Strains

For in vitro Caco-2 cell invasion assay, Lm CIP101821 donated by Collection de l'Institut Pasteur was used. This is a human origin epidemic strain and belongs to the serotype 4b group [20]. In a preliminary study, this strain showed the highest invasion activity into Caco-2 cells. It was precultured in brain heart infusion broth (BHI; Becton Dickinson, Sparks, MD, USA) at 37 °C for 20 h (stationary growth phase). Viable counts of the culture were about 9 log CFU/ml.

Preparation of Ethanol Extract and Crude Soluble Polysaccharide

A dried kombu product was purchased from a retail shop in Hakodate, Hokkaido, Japan. Dried ganiashi was kindly gifted from Kyosei Pharmaceutical Co. (Sapporo, Hokkaido, Japan). These dried products were milled and sieved through 0.5×0.5 mm mesh. The powder (2 g) was extracted with ten volumes of ethanol under shaking (120 rpm) for 1 h at room temperature (22 °C). After centrifugation at 3,000×g for 10 min at 4 °C, the supernatant was collected as EE solution. The EE was dried using a rotary evaporator, for subsequent use in

determining total phenolic content, as well as in human enterocyte Caco-2 and murine macrophage RAW 264.7 cell assays. Then, the ethanol insoluble residue was suspended in 40-ml distilled water with shaking for 3 h. After the centrifugation, crude SPS in the supernatant was collected by an ethanol precipitation method as previously reported [9].

Viscosity of the SPS solution (50 mg dried sample equivalent (DSEq)/ml) was measured using a viscometer (Viscomate VM 1G, Yamaichi Electronics, Co., Tokyo, Japan) as previously reported [6]. Analysis of total phenolic content using Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, and ferrous-reducing power was carried out according to our previous report [11, 21]. (+)-Catechin was used as an antioxidative positive control.

Antibacterial activity of SPS and EE against Lm was determined using an agar spot test [22]. Briefly, the sample solutions were filtered through a 0.2 μm filter. Lm was preincubated in BHI broth at 37 °C for 20 h, and 0.05 ml of the culture was spread on BHI agar. After 3 min, the filtered sample solutions (0.02 ml) were spotted on the agar plates. After 24 h of incubation at 37 °C, inhibition zones from the sample solutions were visually observed.

Caco-2 Cell Invasion Assay

Caco-2 cell invasion assay was carried out according to the method previously reported [22]. For undifferentiated Caco-2 cell assay, a confluent cell monolayer of Caco-2 ATCC No. HTB-37 cells were prepared using the Preset Vecell system (Vessels Co., Kitakyushu, Japan). The cells were seeded onto membrane bottomed wells at a density of about 5 log cells/well and incubated in Eagle's Minimum Essential Medium (Sigma-Aldrich Co., St. Louis, MO, USA) containing 10 % (v/v) fetal bovine serum (Sigma-Aldrich) and 1 % (v/v) amino acid solution (DS Pharma Biomedical Co., Suita, Japan) for 72 h at 37 °C under 5 % CO₂. After aspirating the medium, 0.4 ml of fresh medium containing 0.05 mg DSEq of SPS or EE/ml was added ($n=4$). After 24 h incubation, 0.4 ml of fresh medium containing about 7 log CFU/ml Lm was placed in the wells. Following 2 h incubation at 37 °C, bacterial cells that did not adhere to Caco-2 cells were washed away with phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan). The cells were incubated at 37 °C for 1 h with the medium containing 50 $\mu\text{g}/\text{ml}$ gentamicin (Sigma-Aldrich) to kill extracellular adherent bacteria. Then, the cells were washed three times with PBS and lysed by maintaining them for 10 min in cold PBS containing 1 % triton X-100 (Wako Pure Chemical). Viable Lm released from the Caco-2 cells was spread on Palcam agar (Merck, Darmstadt, Germany) [23]. For the invasion assay using differentiated cells, Caco-2 cells were incubated for 13 days, and the medium was changed every third day. Then, the SPS and EE treatment and Lm invasion assay were carried out as above.

NO Production from Intact and LPS-Activated Murine Macrophage RAW 264.7 Cells

RAW 264.7 cell line ATCC TIB-71 was used as a macrophage cell model in this study as same as our previous report [24]. The murine macrophage cells were maintained in RPMI 1640 medium (Life Technologies, Tokyo, Japan), supplemented with 10 % w/w heat-inactivated fetal bovine serum at 37 °C in 5 % CO₂. Lipopolysaccharide (LPS), a macrophage activator, from *Escherichia coli* O111 B4 (Sigma-Aldrich) was dissolved in the same medium (4 mg/ml). After counting the cells using a hemocytometer, aliquots (0.1 ml; 1×10^6 cells/ml) of the cell suspension were added to a 96-well microplate. After 3 h incubation, to allow for cell attachment, the medium culture was replaced with fresh identical medium without (control) and with 0.05 mg DSEq of SPS or EE/ml and was incubated for 2 h. Then, 0.01 ml of the LPS solution (final concentration 0.36 mg/ml) was added and incubated for

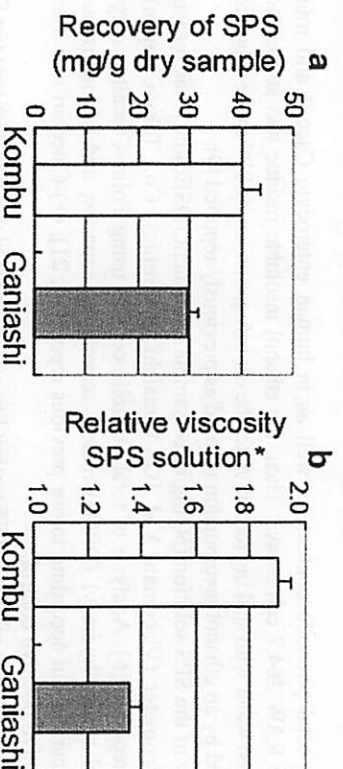


Fig. 1 Recovery of soluble polysaccharide from frond (kombu) and holdfast (ganiashi) parts of *L. japonica* (a) and their relative viscosity (b). *Asterisk*: 50 mg dry sample equivalent/L. Values are expressed as means and SD ($n=3$)

18 h. NO concentration in the culture was determined using Griess reagent (Wako Pure Chemical, Osaka, Japan) as previously reported [25].

Statistical Analysis

Statistical analysis for the Caco-2 and RAW 264.7 cell assays was performed using EXCEL Statistic 5.0 software (Esunmi Co., Ltd., Tokyo, Japan). One-way ANOVA was used to assess overall variation and individual groups were compared to the control group using the Dunnett's post hoc test. A $P<0.05$ threshold was used to determine significant differences.

Results and Discussion

SPS Recovery and Antioxidant Properties of EE

SPS recovery from the kombu and ganiashi water extract solutions was about 40 mg and 30 mg/g dry sample, respectively (Fig. 1a). As shown in Fig. 1b, relative viscosity (distilled water=1.0) of the SPS solution (50 mg DSEq/ml) of kombu (1.91) was higher than that of ganiashi (1.35). Total phenolic compound content in kombu and ganiashi EE was about 40 and 130 nmol catechin equivalent (CatEq)/g dry sample, respectively (Fig. 2a). From the

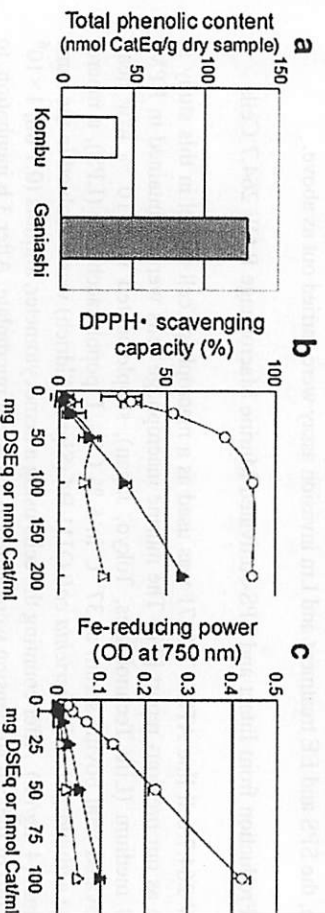


Fig. 2 Total phenolic content (a), DPPH radical scavenging capacity (b), and ferrous-reducing power (c) of ethanol extract from kombu (*open triangles*) and ganiashi (*closed triangles*), and catechin (*open circles*). Values are expressed as means and SD ($n=3$)

IC50 values, DPPH radical scavenging capacity of kombu and ganiashi EE are <30 and about 150 nmol CatEq/g dry product, respectively (Fig. 2b). Fe-reducing power of kombu and ganiashi EE was about 60 and 200 nmol CatEq/g dry sample, respectively (Fig. 2c). These antioxidant activities were correlated with the total phenolic compound content. SPS and EE did not show antibacterial activity against *Lm* in the agar spot test (data not shown).

Effect of Kombu and Ganiashi SPS and EE on *L. monocytogenes* Caco-2 Invasion

Relative invasion of *Lm* into undifferentiated (4 day incubation) and differentiated (14 day incubation) human intestinal epithelial Caco-2 cells is shown in Fig. 3. The invasion efficiency of *Lm* into the intact Caco-2 cells was about 5 log CFU/well. The invasion to

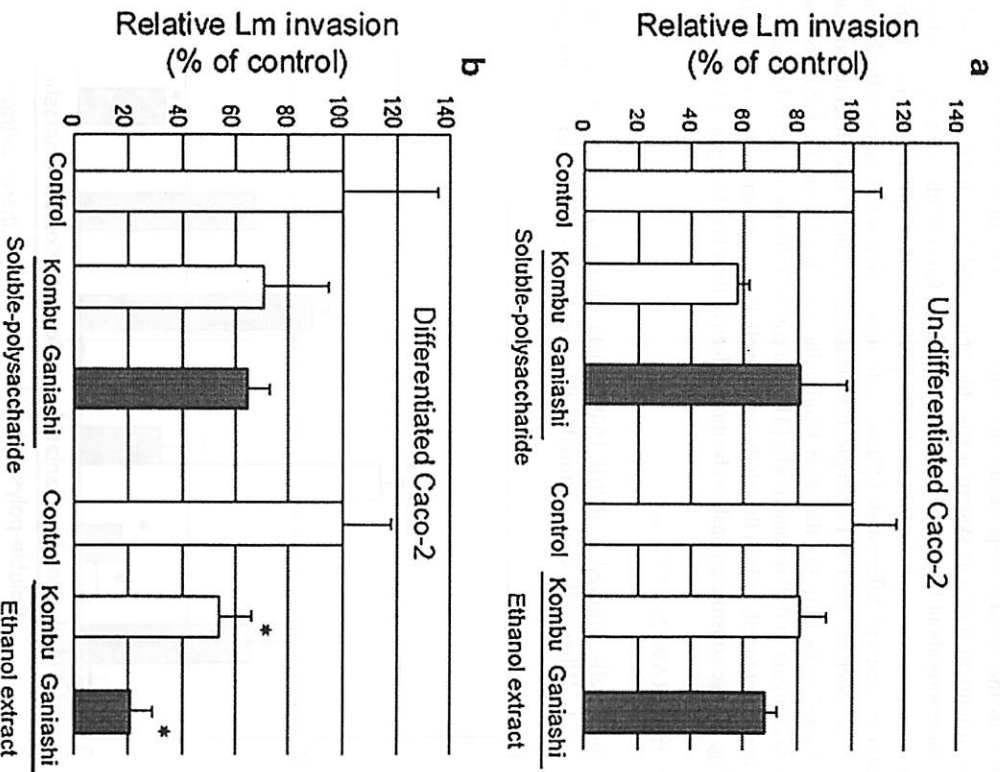


Fig. 3 Effect of soluble polysaccharide and ethanol extract from kombu and ganiashi parts on *L. monocytogenes* invasion into human enterocyte-like undifferentiated (a) and differentiated (b) Caco-2 cells; 0.05 mg DSEq/ml of the samples was added. Values are expressed as means and SEM ($n=4$). Asterisk: Significantly lower than control ($*p<0.05$)

undifferentiated Caco-2 cells tended to be suppressed (by about 20–45 %) by the SPS and EE samples, though the effects was not significant (Fig. 3a). In the case of differentiated Caco-2 cells, the cell invasion tended to be suppressed (about 30–35 %) by the sample SPS solutions (Fig. 3b). Furthermore, kombu and ganiashi EE solutions significantly suppressed cell invasion by about 50 and 80 %, respectively.

Although antibacterial effect of various algae had been reported [11], that of SPS and EE of kombu and ganiashi was not shown. Therefore, it is considered that the inhibitory effect of the samples on the Lm invasion was not caused by the antibacterial activity. So, it can be considered that the EE solutions affected on immune system of Caco-2 cells. For examples, Hummel et al. [26] reported that probiotic lactobacilli improve epithelial barrier function.

In this study, the results in undifferentiated Caco-2 cells are different from those of differentiated cells (Fig. 3). Differentiated Caco-2 cells might have an apical brush border and basolateral surfaces similar to the intestinal epithelial monolayer [27]. The differentiated Caco-2 cell monolayer has a nutrient transport system across the apical and basolateral membranes [28]. There are several reports about Lm invasion to undifferentiated Caco-2 cells with sigma B factors and virulence-related genes, such as *InlA* and *prfA* [29]. However, differences in sensitivity between undifferentiated and differentiated Caco-2 cells to infection with Lm and other bacterial pathogens have also been reported. For example, Lm invasion is higher in undifferentiated than in differentiated Caco-2 cells [30]. On the other hand, adhesion of a human *Lactobacillus acidophilus* isolate was higher in differentiated cells [31]. We consider that the result in differentiated, rather than in undifferentiated, cells in this study reflect well this phenomenon in vivo. Therefore, as shown in Fig. 3b, we consider ganiashi to be more effective than kombu against Lm infection.

NO Secretion from RAW 264.7 Cells

Intact RAW 264.7 cells cultured in RPMI 1640 generated only a little NO in the culture supernatant (Fig. 4). When cells were cultured with ganiashi SPS without LPS, a significant

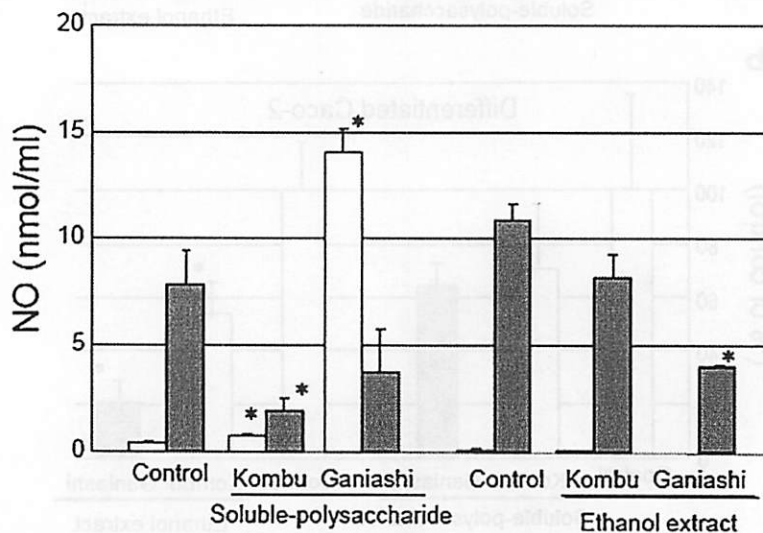


Fig. 4 Effect of soluble polysaccharide and ethanol extract from kombu and ganiashi on NO production of intact (open column) and lipopolysaccharide-activated (closed column) murine macrophage RAW 264.7 cells; 0.05 mg DSEq/ml of the samples was added. Values are expressed as means and SEM ($n=4$). Asterisk: Significantly lower than control ($*p<0.05$)

amount of NO was produced (about 14 nmol/ml). Though kombu SPS also increased NO secretion, it was little (1 nmol/ml). Neither kombu EE nor ganiashi EE exhibited macrophage cell activation in the absence of LPS. On the other hand, cells cultured with LPS (0.4 mg/ml), a macrophage activator, generated 8–11 nmol/ml NO. In this case, LPS-induced NO production was significantly suppressed by kombu SPS (from about 8 to 2 nmol/ml) and by ganiashi EE (from 11 to 4 nmol/ml).

As mentioned above, NO is a highly reactive free radical [17]. NO may play an important role in the pathophysiology of various diseases and its role in macrophage toxicity is well-established [18]. Furthermore, NO acts as a cytotoxic agent in the immunological interaction between invading microorganisms and macrophages [19]. In this study, the increase in NO secretion in the absence of LPS is thought to be caused by the immune-promoting activity of ganiashi SPS (Fig. 4). The immune-promoting activity of brown algal polysaccharides, particularly in fucoidan, is well-known [32]. On the other hand, suppression of LPS-stimulated NO secretion might be due to the anti-inflammatory effect of both the SPS and EE of kombu and ganiashi. There are reports that the anti-inflammatory activity of various brown algae is related to polyphenol compounds [10], as well as soluble polysaccharides. The polyphenol compounds in various algae are also regarded as antioxidants, exhibiting antiaging, antitumor, and antibacterial [6, 11, 33].

From the results in this study, it can be considered that ganiashi, which is a by-product of kombu cultivation, is useful for suppression of Lm invasion and infection. Interestingly, ganiashi has a twofold effect on macrophage cell immune responses: the immune-promoting activity of SPS and the anti-inflammatory effect of the low-molecular weight compounds in EE. Further studies, such as the induction of cytokine-related immune activation against Lm and other pathogen by kombu, ganiashi, and their components, are now in progress.

Conclusions

To determine the protective effects of dried *L. japonica* products against Lm infection and bacterial inflammation, the inhibitory effects of crude SPS and EE solutions of frond (kombu) and holdfast (ganiashi) parts on Lm invasion into Caco-2 cells and on NO production of murine macrophage RAW 264.7 cells were investigated. Ganiashi EE and kombu EE decreased Lm invasion into differentiated Caco-2 cells. Ganiashi SPS significantly increased the NO production of intact RAW 264.7 cells. On the other hand, NO production of LPS-activated RAW 264.7 cells was significantly suppressed by kombu SPS and ganiashi EE. These results suggest that *L. japonica* intake, particularly with respect to ganiashi, might prevent *L. monocytogenes* enterogastric invasion and infection.

Acknowledgments This research was supported by the Ministry of Education, Culture, Sports, Science and Technology in Japan, Grant-in-Aid for Scientific Research (C), 22580221, 2010.

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