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ORIGINAL ARTICLE

Fermented food Tempeh induces interleukin 12 and enhances macrophage phagocytosis

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Abstract

It is known that lactic acid bacteria induce the IL-12. The IL-12 activates NK cells and promotes the production of IFN-γ. The IFN-γ activates macrophages resulting in enhanced phagocytosis and bactericidal activity. We have been investigating fermented foods that activate the immune function. In this study, we investigated the IL-12 inducibility of fermented foods using the specific antibody. Fermented soybean foods such as Tempeh and Natto are attracting attention in terms of nutrition, functionality, and food problems. In this study, Tempeh induced 1,080 μg/ml of IL-12, and IFN-γ associated with the induction of IL-12 was also induced at 682 μg/ml. This was more than twice the induced intensity of PBS. On the contrary, Natto hardly induced IL-12 and IFN-γ. Tempeh also accelerated phagocytosis of the macrophage THP-1 cells. In this study, it was found that the fermented soybean-derived food, Tempeh, has a function of activating the immune function. This is the first report that Tempeh activates innate immunity.

Practical applications

Tempeh, a fermented soybean food induced the IL-12 and IFN-γ production and the increase of macrophage phagocytosis in this study suggested a new function to enhance immunity. Tempeh is also expected to be effective in preventing lifestyle diseases. Fermented soybean products of Tempeh was considered to be a very useful health food for the problems of modern society such as maintaining health by eating, improving immunity, and ingesting vegetable protein due to diversifying food.

KEYWORDS

fermented foods, IFN-γ, IL-12, Natto, phagocytosis, Tempeh

1 | INTRODUCTION

Cytokines play an important role in maintaining the homeostasis of hematopoiesis, cell proliferation and differentiation, as well as the immune system consisting of innate and adaptive immunity in the body. Interleukin-12 (IL-12) is a member of the IL-12 family of cytokines. IL-12, which is secreted by phagocytic cells in response to pathogens, is a heterodimer protein of two subunits (p35 and p40) that acts primarily during the induction of Interferon-γ (IFN-γ) via the signal transducer and activator of transcription 4 (STAT4) in T and natural killer (NK) cells (Guo et al., 2019). IL-12 is a cytokine that strongly induces the Th1 response. Activated macrophages by IFN-γ enhance phagocytosis and bactericidal activity (Masuda et al., 2010).

There are many reports about the induction of IL-12 by *Lactobacillus* (De Andrés et al., 2018, Gao et al., 2019, Lee et al., 2017, Luongo et al., 2017, Park et al., 2019). It has been reported that heat-killed *Lactobacillus plantarum* L-137 (HK L-137) enhances IFN-γ production through the induction of IL-12 (Nakai et al., 2019). It has also been reported that heat-treated *Lactobacillus plantarum* increases the immune responses through activation of NK cells and macrophages based on in vivo and in vitro models (Moon et al., 2019).

Lactic acid bacteria are microorganisms used in various fermented foods, and miso made from soybeans has been reported as a fermented food having an immunostimulatory effect (Nagao, 2018). However, although various other microorganisms are involved in fermented foods, there are few reports of IL-12 induction by microorganisms other than lactic acid bacteria. Typical microorganisms are molds such as *Aspergillus*, *Rhizopus*, and *R. oligosporus* is used for the production of tempeh made from soybeans. Therefore, we compared the expression-inducing ability of IL-12 between Natto made from soybeans and Tempeh. Tempeh is one of the sources of protein in various traditional Indonesian fermented foods. Tempeh is made from soybeans fermented with *Rhizopus* (Ahnan-Winarno et al., 2021). When boiled soybeans are wrapped in banana leaves, the Tempeh bacteria on the banana leaves promote fermentation and complete the Tempeh. Tempeh is said to be similar to Natto, but it does not have the strong smell and taste of Natto. Natto is a Japanese fermented food made from soybeans with *Bacillus subtilis var. Natto* in *bacillus* or *subtilis* Natto (Sella et al., 2021).

Soybeans are also attracting attention as an alternative protein to animal protein, and if we can find added value in the immunostimulating activity of Tempeh, which is a fermented soybean food, we can expect it to contribute not only to dietary health but also to the SDGs (Sustainable Developmental Goals). Sirtori et al. reported that soy protein is effective for patients with hypercholesterolemia due to its high cholesterol-lowering effect (Sirtori et al., 1979). Foods that are fermented from soybeans include miso, soy sauce, Tempeh, and Natto. However, miso and soy sauce have a high salt content, and thus large amounts of them can affect health. Tempeh and Natto are fermented foods that can be consumed directly from fermented soybeans, thus providing the rich nutrients of soybeans as well.

In this study, we analyzed the IL-12 and IFN-γ inducing properties of Tempeh, which have not been reported before, and compared it with Natto, which has different production methods and bacteria species. Since there are few reports of IL-12 induction by bacteria other than lactic acid bacteria, we analyzed the IL-12 and IFN-γ inducibility of Tempeh and Natto.

2 | MATERIAL & METHOD

2.1 | Materials

2.1.1 | *Rhizopus oligosporus* and *Aspergillus oryzae*

Rhizopus oligosporus and *Aspergillus oryzae* were obtained from Akita Konno Co., Ltd. (Kariwano, Akita, Japan). They were inoculated into PDA (Potato Dextrose Agar) medium and incubated at 27°C for about 5 days until sporulation. After sporulation, the culture was kept at room temperature. Inoculated into a PD (Potato Dextrose) liquid medium that had been autoclaved at 121°C for 20 min and shake-cultured at 30°C for 4 days. After culture, autoclave sterilization was performed at 121°C for 20 min and dead cells were collected. The medium components were removed by washing with sterile distilled water several times, and the medium was lyophilized.

2.1.2 | EC12 (*Enterococcus faecalis*)

EC12 was obtained from Combi Co., Ltd. Japan (Asakusa, Tokyo, Japan).

2.1.3 | Soybeans

Soybeans were purchased from Buyou Foods Co., Ltd. (Marunouchi, Tokyo, Japan). The washed soybeans were soaked overnight to prepare the dehulled soybeans, then steamed and sterilized for 60 min. The prepared soybeans were sterilized in an autoclave at 121°C for 20 min dried at 100°C for 24 hr and crushed with a mill.

2.1.4 | Natto

Commercially available Natto (Yamadafoods Co., Ltd., Misato, Akita, Japan) was autoclaved at 121°C for 20 min, dried at 100°C for 24 hr, and crushed with a mill.

2.1.5 | Tempeh

The washed soybeans were soaked overnight to prepare the dehulled soybeans, which were sterilized by steaming for 60 min. After cooling to about 40°C, *R. oligosporus* were inoculated and cultured in a perforated plastic bag at 30°C for 24 hr to produce soybean Tempeh. The prepared Tempeh was sterilized in an autoclave at 121°C for 20 min dried at 100°C for 24 hr and crushed with a mill.

2.2 | Cell culture and cell differentiation

Caco2 cells derived from human colon cancer (kindly provided from Professor Dr. Shinichi Yokota, Sapporo Medical University, Japan) and THP-1 derived from human acute monocytic leukemia (obtained from RIKEN Cell Bank, Japan) were used in this study. Caco2 cells were cultured in a 5% CO_2 incubator in DMEM medium (Dulbecco's converted Eagle's medium, Nacalai Tesque Inc., Japan) containing 5% FBS (Biological industries, USA) and 0.2% penicillin streptomycin. THP-1 cells were cultured in RPMI 1640 medium (Nacalai Tesque Inc., Japan) containing 10% FBS in a 5% CO_2 incubator. Caco2 cells

were seeded on Cell Culture Inserts (Millicell, 6-Well Hanging Inserts 0.4 μm PET, Merck, German) and cultured. The cells were differentiated into intestinal epithelium-like in a medium containing 5.0 mM butyric acid for 4 days. The cell differentiation was measured transepithelial electrical resistance (TEER) using Millicell-ERS (Merk) and used differentiated cell with a TEER value above 400 Ωx cm^2. THP-1 cells were seeded in multiwell (6 well, Falcon, USA), and cultured and differentiated into macrophage-like for 3 days in a medium containing 1,000-fold diluted PMA (Phorbol12-myristate13-acetate) and 2,600-fold diluted Cholecalciferol (Tokyo Chemical Industry Co., Ltd., Japan). After each differentiation, Caco2 and THP-1 cells were co-cultured in Transwell.

2.3 | Addition of samples to cells cultured in the Transwell system and preparation of medium samples for analysis of IL-12 and IFN-γ

Each *R. oligosporus*, Tempeh, and soybeans was suspended in PBS and sterilized at 100°C for 5 min. The samples were added to the upper compartment side of Transwell system at a final concentration of 0.5 mg/ml and stimulated at 37°C in the presence of 5% CO_2 for 24, 48, 72, and 96 hr. After incubation, the medium on the lower compartment side was collected, and after removing impurities by centrifugation at 12,100 g for 10 min, 10% volume of 100% TCA was added to medium supernatant and precipitated the protein for 30 min at on ice. After centrifugation at 12,100 g for 20 min, the supernatant was removed, and the precipitate was dissolved in 2X SDS-sample buffer (including 10% β-mercaptoethanol) for concentration (10-fold concentration). The sample was heat-treated at 100°C for 5 min to prepare an analyzing sample. A non-stimulated group using PBS instead of the sample was used as a control. Samples were electrophoresed on SDS-PAGE, followed by immunoblotting using an antibody against IL-12p35, IFN-γ, or β-actin. Total IL-12 and IFN-γ were calculated using Image-J software. Each *R. oligosporus*, Tempeh, *A. oryzae*, soybeans, Natto, Lactic acid bacteria (*Lactobacillus plantarum*), and EC12 (*Enterococcus faecalis*) powder was suspended in PBS and sterilized at 100°C for 5 min. The samples were added to the upper compartment side at a final concentration of 0.5 mg/ml and stimulated at 37°C in the presence of 5% CO_2 for 24 hr. Total IL-12 and IFN-γ were investigated as described above.

2.4 | Analysis of *E. coli* phagocytosis using THP-1 cells

Escherichia coli (E. coli) TOP-10 were labeled using 0.1 mg/ml of FITC (fluorescein isothiocyanate, Dojin) and mild shake at room temperature for 30 min under shading. After shaking, TOP-10 were centrifuged at 18,800 g for 15 min at 4°C. Supernatant was discarded and FITC labeled-*E. coli* were washed with PBS until the solution becomes colorless. Caco2 and THP-1 cells were differentiated and co-cultured in the Transwell system as described above. Set the cover glass on the six-well dish. After 72 hr of cell differentiation, the cells were replaced with RPMI1640 serum-free medium using the Transwell system. After adding the *R. oligosporus* and Tempeh (final conc. 0.5 mg/ml) to Transwell system, the cells were cultured for 24 hr. FITC labeled-*E. coli* was added to the THP-1 (number ratio was 500:1) cells and cultured for 15 min at CO_2 incubator. After incubation, culture medium was collected and the cells were washed with PBS three times. The washed PBS was collected and combined to culture medium for measurement of the number of surviving *E. coli* by phagocytosis. Nuclear were stained with DAPI for 30 min. After washing with PBS for three times, phagocytosis of THP-1 cells were analyzed using HS All-in-One Fluorescence Microscope BZ-8000 (KEYENCE).

2.5 | Measurement of the survived *E. coli* by phagocytosis

The number of *E. coli* that were not phagocytosed as described above was determined by diluting the medium 1×10^6 times, streaking on LB (Lysogeny Broth) medium agar plates, and incubating at 37°C for 16 hr. The number of colonies was counted.

2.6 | SDS/PAGE and Immunoblotting

SDS/PAGE and Immunoblotting were followed Laemmli (Laemmli, 1970) and Towbin's methods (Towbin et al., 1979), respectively. Samples were analyzed by SDS/PAGE, followed by immunoblotting. The PVDF membrane was reacted with an IL-12p35 antibody (Hosaka et al., 2021) (diluted 500 times) with an anti-rabbit AP IgG (BioRad, diluted 10,000 times) or an anti-rabbit HRP IgG (Biorad, diluted 15,000 times). Samples were treated with BCIP-NBT solution (Nacalai Tesque) or an ECL Plus Western Blotting Detection System (Cytiva). Image analysis was performed using ChemiDoc XRS plus (BioRad).

2.7 | Data analysis

2.7.1 | IL-12 and IFN-γ

The bands obtained by immunoblotting were analyzed by ImageJ. As an internal standard, β-actin was analyzed in the same way and corrected for the amount of protein. The mean value and standard deviation were calculated. The superiority of the differences was analyzed by Student's *t* test.

2.7.2 | Phagocytosis

The phagocytosis of THP-1 cells was analyzed using the HS All-in-One Fluorescence Microscope BZ-8000 (KEYENCE). For surviving

E. coli, CFU was measured at $n = 3$ and the mean value was adopted. The superiority of the differences was analyzed by Student's *t* test.

3 | RESULTS

3.1 | Specificity of antibodies against IL-12 and IFN-γ

We constructed, then purified human IL-12p35 and IFN-γ using the *E. coli* expression system (Hosaka et al., 2021). We also immunized the purified proteins to rabbit and obtained antiserum against IL-12p35 and IFN-γ. The IL-12p35 antibody was able to detect IL-12 which was stimulated by *Lactobacillus* and secreted into Caco2 cell medium as a single protein band (Figure 1). The IFN-γ antibody also detected IFN-γ as a single protein band.

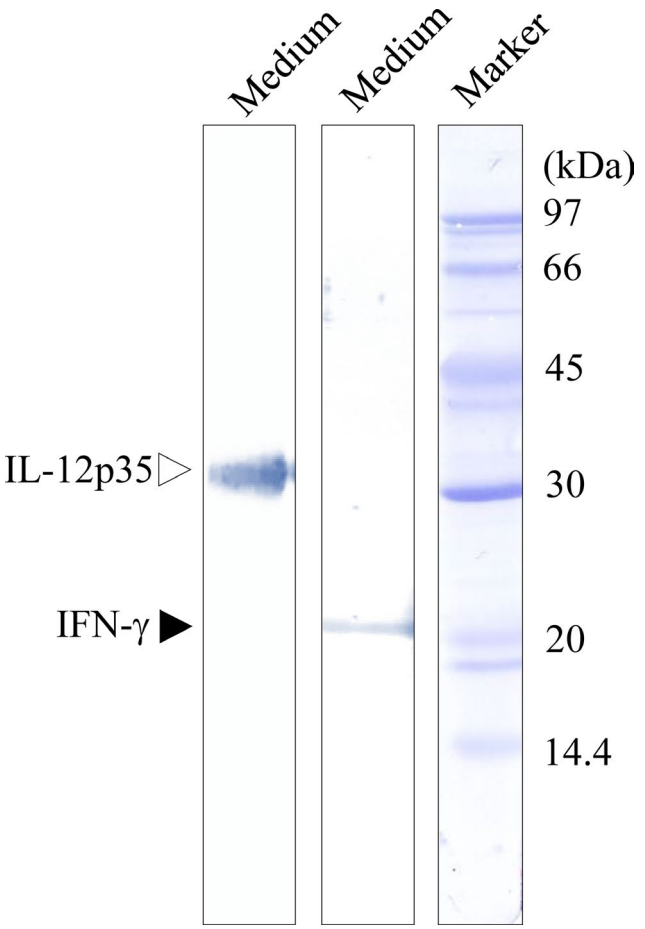

FIGURE 1 Antibodies against IL-12 and IFN-γ. Lactic acid bacterium-administered Caco2 cell culture medium was electrophoresed on SDS-PAGE, followed by immunoblotting with a polyclonal antibodies IL-12p35 and IFN-γ. Each antibody could be detected as a single protein band

3.2 | IL-12 inducibility

The optimal IL-12 induction time when Tempeh was added to Caco2 cells was determined. As a result of determining the optimum stimulation time, Tempeh produced the highest amount of IL-12 in 24 to 48 hr, and *R. oligosporus* produced the highest amount in 48 hr (Figure 2a,b). Since both tended to sharply decrease at 72 hr in this study, the ability to induce IL-12 production was evaluated at a stimulation time of 24 hr, similar to lactic acid bacteria. Since the IL-12 production-inducing ability tended to decrease sharply at 72 hr in this study, the IL-12 inducibility was evaluated at the stimulation time of 24 hr.

Next, we analyzed the IL-12 inducibility 24 hr after the addition of *R. oligosporus*, Tempeh, *A. oryzae*, boiled beans, and Natto. As shown in Figure 2c,d, the IL-12 inducibility of *R. oligosporus* was about 2.2 times that of *A. oryzae*, which is the same filamentous fungus. It was confirmed that Tempeh has an IL-12 inducibility about 3.4 times that of raw soybeans materials. Tempeh induced IL-12 about 2.6 times that of Natto, which is the same fermented soybean food. These results suggest that *R. oligosporus* and Tempeh have a high IL-12 production-inducing ability. We also investigated the IL-12 inducibility of *R. oligosporus*, Tempeh, soybeans, lactic acid bacteria (*Lactobacillus plantarum*), and EC12 (*Enterococcus faecalis*). As shown in Figure 2e,f, the IL-12 inducibility of Tempeh was about 1.5 times higher than that of lactic acid bacteria and EC12. The *R. oligosporus* was almost the same as or slightly lower than that of lactic acid bacteria and EC12. Significance of difference was analyzed by Student's *t* test. $^*p < .05$.

3.3 | IFN-γ inducibility

IL-12 is a potent regulator of cell-mediated immunity and induces the production of IFN-γ by NK and T cells. We investigated the IFN-γ inducibility of *R. oligosporus*, Tempeh, *A. oryzae*, soybeans, Natto and PBS. As shown in Figure 3a,b. In all the test samples, higher IFN-γ inducibility than PBS (non-stimulation) was confirmed. Among them, *R. oligosporus* and Tempeh tended to be high, and induced the production of IFN-γ, which was about 2.3 to 2.5 times than that of PBS. These results were similar to the IL-12 inducible results (Figure 2d). Significance of difference was analyzed by Student's *t* test. $^*p < .05$.

3.4 | Phagocytosis

Macrophages produce IL-12 and differentiate naive T cells into Th1 cells. These Th1 cells produce IFN-γ, and the resident macrophages are activated resulting in bactericidal activity. We labeled *E. coli* with FITC and added it to the THP-1 cells. As shown in Figure 4a, the macrophage phagocytosed the FITC-labeled *E. coli*. The green circles and lines in the THP-1 cells were phagocytosed *E. coli*. The nuclear was stained a blue by DAPI. Some phagocytosed *E. coli* were detected in the PBS. On the contrary, many phagocytosed *E. coli* were detected in *R. oligosporus* and Tempeh. We cultured the surviving *E. coli* that

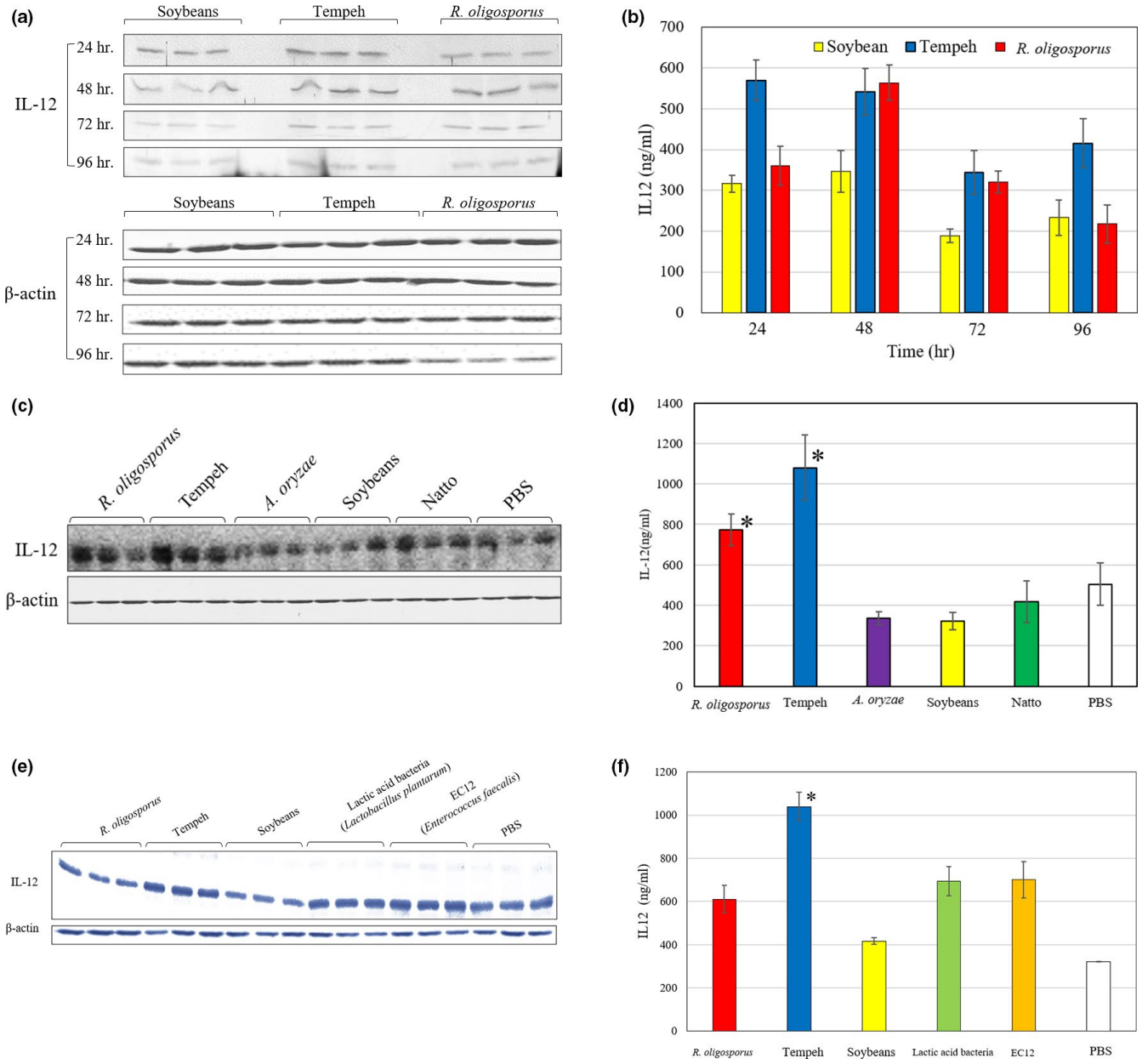

FIGURE 2 IL-12 inducibility. IL-12 induction ability of fermented foods, bacteria, and soybean were analyzed on immunoblotting using IL-12p35 antibody. (a) Soybeans, Tempeh, and *R. oligosporus* were added to the Transwell system as described in the "MATERIALS AND METHODS". Immunoblotting using an antibody against IL-12p35 and β-actin ($n = 3$) at 24, 48, 72, and 96 hr. (b) IL-12 inducibility of soybeans, Tempeh, and *R. oligosporus* at 24, 48, 72, and 96 hr. Total IL-12 was calculated using immunoblotting area (a) and calibration curve (Ref. 13). (c) *R. oligosporus*, Tempeh, *A. oryzae*, soybeans, and Natto powder were suspended in PBS and sterilized at 100°C for 5 min. The samples were added to cells cultured in the Transwell system as described in the "Materials and Methods." Samples were separated by SDS-PAGE, followed by immunoblotting using the antibodies against IL-12p35 and β-actin ($n = 3$). (d) Total IL-12 was calculated immunoblotting area using Image-J software (c) and calibration curve (Hosaka et al., 2021, *American Journal of Molecular Biology*. 11(2). 29–37. https://www.scirp.org/journal/paperinformation.aspx?paperid=107559). *$p < .05$, compared with PBS. (e) *R. oligosporus*, Tempeh, soybeans, Lactic acid bacteria (*Lactobacillus plantarum*), EC12 (*Enterococcus faecalis*) powder were suspended in PBS and sterilized at 100°C for 5 min. The samples were added to cells cultured in the Transwell system as described in the "MATERIALS AND METHODS." Samples were separated by SDS-PAGE, followed by immunoblotting using the antibodies against IL-12p35 and β-actin ($n = 3$). (f) Total IL-12 was calculated immunoblotting area (e) using Image-J software and calibration curve (Hosaka et al., 2021, *American Journal of Molecular Biology*. 11(2). 29–37. https://www.scirp.org/journal/paperinformation.aspx?paperid=107559). *$p < .05$, compared with EC12

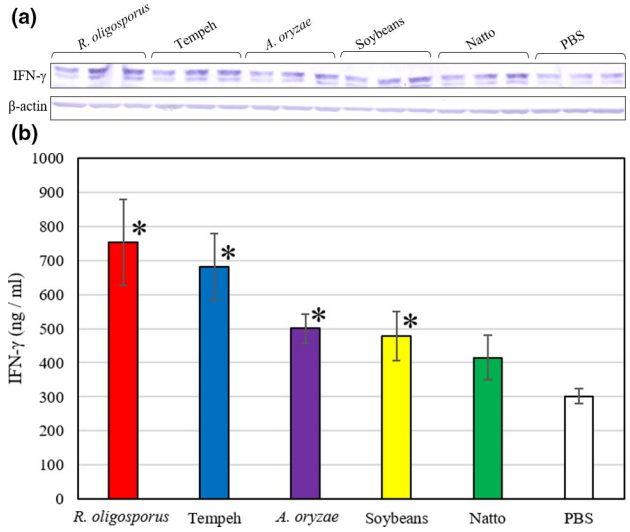

FIGURE 3 IFN-γ inducibility. (a) IFN-γ induction ability of fermented foods, bacteria, and soybean were analyzed on immunoblotting using IFN-γ antibody. *R. oligosporus*, Tempeh, *A. oryzae*, soybeans, and Natto powder were suspended in PBS and sterilized at 100°C for 5 min. The samples were added to cells cultured in the Transwell system as described in the "Materials and Methods." Samples were separated by SDS-PAGE, followed by immunoblotting using the antibodies against IFN-γ and β-actin ($n = 3$). (b) Total IFN-γ was calculated immunoblotting area (a) using Image-J software and calibration curve (Hosaka et al., 2021, *American Journal of Molecular Biology*. 11(2). 29–37. https://www.scirp.org/journal/paperinformation.aspx?paperid=107559). *$p < .05$, compared with PBS

did not undergo phagocytosis by the macrophages on LB agar medium plates (Figure 4b). The number of surviving *E. coli* in *R. oligosporus* was lower than the control and even lower in Tempeh. These results correlated with the IL-12 and IFN-γ inducibility (Figures 2d and 3b). Significance of difference was analyzed by Student's *t* test. *$p < .05$.

4 | DISCUSSION

One of the cytokines, IL-12 (p40/p35), was identified as an NKSF (natural killer-stimulating factor) (Kobayashi et al., 1989) and CLMF (cytotoxic lymphocyte maturation factor) that activates CTL (cytotoxic T lymphocyte; Gately et al., 1991). IL-12 is a cytokine that produces IFN-γ, which is important for inducing protective immunity against infection and tumor immunity, and is essential for inducing the Th1 response. It has been reported that the EBI3 (Epstein-Barr virus-induced gene 3), a molecule whose expression is induced by the EB (Epstein-Barr) virus infection, associates with one of the subunits of IL-12p35 (Devergne et al., 1997). It associates with one of the p40s and was found to be the new cytokine IL-23 (Niedobitek et al., 2002; Oppmann et al., 2000; Tong et al., 2010). Until then, IL-12 (p40/p35) was used in EAE (experimental autoimmune encephalomyelitis) and CIA (collagen-induced arthritis) using antibodies against p40 and p40 gene-deficient mice. It was revealed that the onset of such a tissue-specific autoimmunity was actually caused by IL-23, which shares p40 (Cua et al., 2003; Marafini & Monteleone, 2021). This was a major breakthrough leading to the subsequent discovery of new inflammatory Th17 cells that produce IL-17. The IL-12, IL-27, IL-35, and IL-39 belong to the IL-12 family. This family comprises IL-12 (IL12p35/IL12p40), IL-23 (IL23p19/IL12p40), IL-27 (IL27p28/Ebi3), IL-35 (IL12p35/Ebi3), and IL-39 (IL23p19/Ebi3; Guo et al., 2019; Vignali & Kuchroo, 2012).

The IL-23 is a pro-inflammatory cytokine and is a key pro-inflammatory cytokine in the development of chronic inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, or psoriasis (Gaen et al., 2014; Schinocca et al., 2021). Increased levels of IL-23 have been found in biopsies from patients with psoriasis, Crohn's disease, and ulcerative colitis (Patel & Kuchroo, 2015; Riquelme et al., 2018; Wu & Smogorzewski, 2021). We searched for IL-12-specific antibodies to analyze the IL-12 inducibility, but basically only antibodies against IL-12p40. When we tried to measure the IL-12 inducibility using the anti-IL-12p40 antibody, not only IL-12 but also IL-23 will be quantified, so that accurate quantification of the IL-12 inducibility is not expected. We have been investigating fermented foods that activate the immune function, but even if we obtain data that a fermented food using the IL-12p40 antibody induces IL-12, in some cases, the fermented food is an inflammatory cytokine. It may have induced IL-23. When using the IL-12p40 antibody, it is difficult to distinguish between IL-12 and IL-23.

IL-35 is a member of the IL-12 family, and IL-12 cytokines have emerged as important regulators of host immunity (Vignali & Kuchroo, 2012). Intense inflammation elicits recruitment of dendritic cells and other antigen-presenting cells that produce IL-35 and/or IL-27, thereby promoting the differentiation of regulatory lymphocytes that curtail exuberant immune responses that cause autoimmune disease (Mirlekar & Pylayeva-Gupta, 2021; Xiang & Xie, 2015). IL-35-induced Treg cells (iTr35) produce more IL-35 (Choi & Leung, 2015), and the Tr1 cells (type 1 T regulatory cells) use IL-35 to suppress the immune response (Zhu & Shan, 2020). Unlike IL-12 and IL-23, IL-35 suppresses inflammatory responses by regulating various cytokines, thereby controlling the STAT signaling (Liu et al., 2019; Mirlekar & Pylayeva-Gupta, 2021). For the IL-12 inducibility by bacteria or fermented foods, there are two methods for measuring the antibody capacity of IL-12, one using the IL-12p40 antibody and the other using the IL-12p35 antibody. When we use the IL-12p40 antibody, we can measure IL-12, and at the same time, measure the inflammatory cytokine IL-23. On the contrary, we are able to analyze the IL-12 using the IL-12p35 antibody. In some cases, the antibody cross-reacts also with IL-35p35. Although we postulated that a fermented food induced IL-12 and thus activated the immune function, the result was actually a fermented food that induces the inflammatory cytokine IL-23. For those of us who are looking for immuno-active fermented foods, the result is exactly the opposite, which is very concerning. In that case, it is still better to include the non-inflammatory cytokine IL-35 in the measurement results.

For the IL-12 measurement, we considered using the IL-12p35 antibody rather than the IL-12p40 antibody. We also decided that it was better to analyze IL-12. Thus, we constructed, then purified

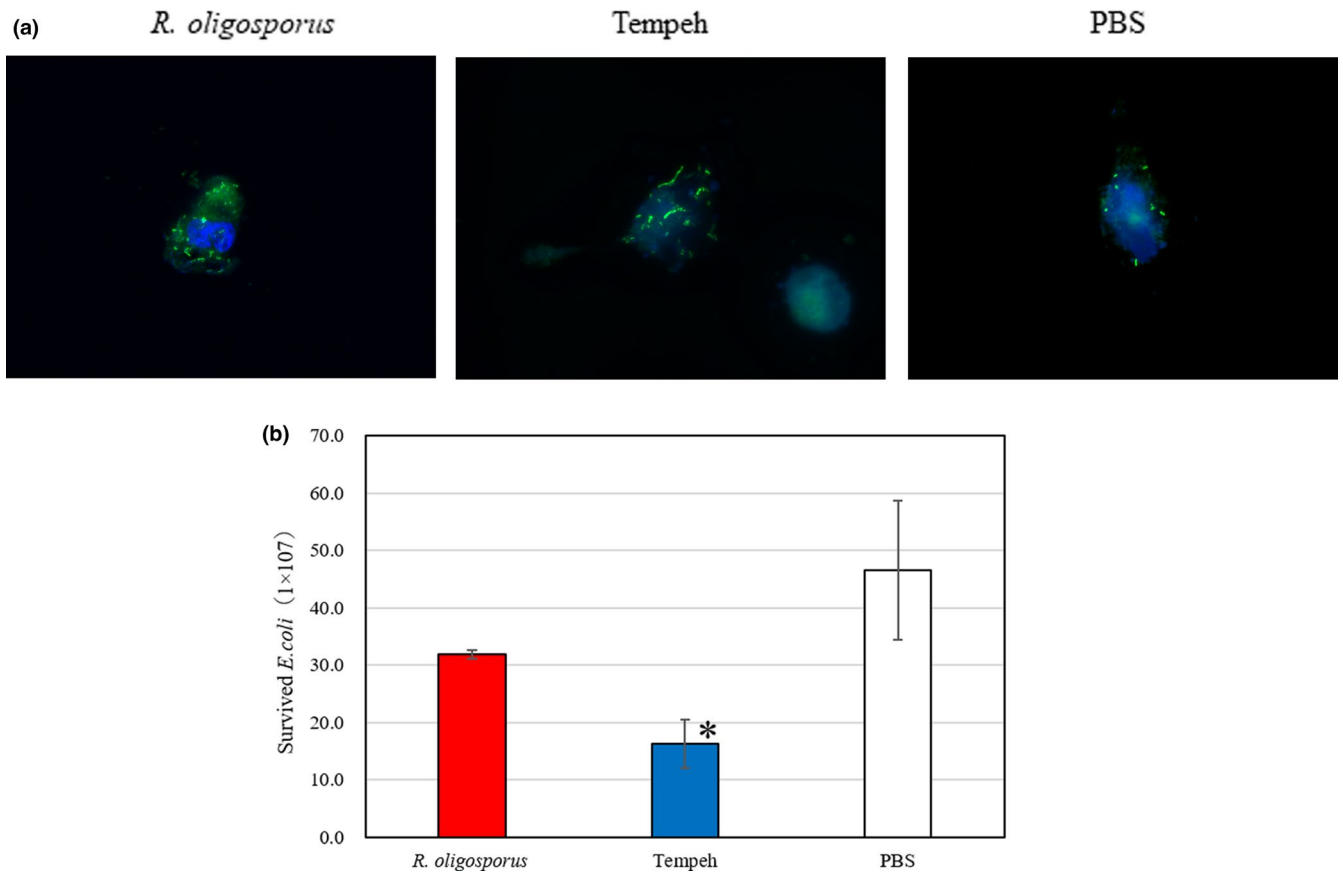

FIGURE 4 Macrophage phagocytosis. FITC-labeled *E. coli* was added to the Transwell system pre-administered with PBS, *R. oligosporus*, and tempeh as described in the "Materials and Methods." (a) Fluorescence microscope image of macrophage phagocytosis. Small green dots and bars are phagocytosed FITC-labeled *E. coli*. Blue is DAPI stained nucleus. (b) Survived *E. coli* in (a) were cultured on LB agar plates and the number of surviving *E. coli* were graphed. $^{*}p < .05$, compared with PBS

IL-12p35, and immunized the protein to rabbit (Hosaka et al., 2021). The antibody against IL-12-p35 detected 35-kDa proteins in the cultured medium as a single protein band (Figure 1). Thus, we obtained a specific antibody against IL-12 and could analyze the IL-12 inducibility of fermented foods. Tempeh strongly induced IL-12 at about 1,000 ng/ml. *R. oligosporus* also induced IL-12 at about 800 ng/ml. Lactic acid bacteria (*Lactobacillus plantarum*) and EC12 (*Enterococcus faecalis*) also induced IL-12, but the inducibility was less than Tempeh with both at 700 ng/ml. Surprisingly, Natto weakly induced IL-12 at about 400 ng/ml the same as *A. oryzae* and soybeans. These are very interesting results. The fermented foods, Natto and Tempeh, are both made from soybeans, Tempeh induces IL-12 and enhances the immune system. Tempeh not only directly induced IL-12, but also IFN-γ. Tempeh also enhanced the phagocytosis of macrophages. Natto indirectly induced IL-12.

Tempeh is rich in soybean isoflavones, and Nagao et al. reported that these isoflavones promote IL-12-production (Nagao, 2018). *R. oligosporus* contains zymosan, a ligand for TLR2, as a cell wall component. Ichikawa et al. reported that expression of TLR2 is essential for the induction of IL-12 by lactic acid bacteria (Ichikawa et al., 2007), and it is assumed that the mechanism of induction of IL-12 production by Tempeh is also TLR2-mediated. Furthermore, *R. oligosporus* is unique among fungi in that it contains chitosan in its cell wall. It has been reported that chitosan activates MyD88 by binding to TLR2 on the surface of mouse macrophages (Da Silva et al., 2008), and MyD88 is deeply involved in the production of IFN-γ, and Lindgren et al. reported that inhibiting homodimerization of MyD88 decreases IFN-γ production. In other words, the induction of IL-12 production by isoflavones and cell wall components in Tempeh is enhanced by chitosan, which may also induce high expression of IFN-γ.

Bacillus subtilis is known to produce catalase and Subtilisin (Loewen & Switala, 1987a, 1987b, 1988), and Hosoi et al. speculated that these are involved in promoting the growth and inhibiting the death of lactic acid bacteria. Thus, since Natto is known to increase lactic acid bacteria, which are intestinal bacteria, it is considered that lactic acid bacteria induce IL-12 and enhance the immune system (Horie et al., 2018; Hosoi et al., 2000; Nishibayashi et al., 2015). Although the IL-12 induction mechanism is different for each substance, both Natto and Tempeh enhance the immune function as healthy foods.

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CONFLICT OF INTEREST

The authors have no conflict of interest. The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Yoshihito Hosaka: Conceptualization; Data curation; Formal analysis; Methodology; Validation; Visualization; Writing-original draft. **Kei Itoh:** Data curation; Formal analysis; Methodology; Validation; Visualization. **Shun Matsutani:** Data curation; Formal analysis; Methodology; Validation; Visualization. **Shinya Kawate:** Data curation; Formal analysis; Validation; Visualization. **Atsuko Miura:** Data curation; Methodology; Validation; Visualization. **Yukaze Mizoura:** Data curation; Formal analysis; Validation; Visualization. **Sayumi Yamada:** Data curation; Formal analysis; Validation; Visualization. **Hiroshi Konno:** Conceptualization; Funding acquisition; Supervision. **Ewa Grave:** Conceptualization; Writing-review & editing. **Koji Nagata:** Conceptualization; Supervision; Writing-review & editing. **Hideki Wakui:** Conceptualization; Supervision; Writing-review & editing. **Hideaki Itoh:** Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Writing-original draft; Writing-review & editing.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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