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Citation	Food Chemistry, 126(4), 1521-1526 https://doi.org/10.1016/j.foodchem.2010.12.063
Issue Date	2011-06-15
Doc URL	https://hdl.handle.net/2115/45764
Type	journal article
File Information	FC126-4_1521-1526.pdf



Immunomodulatory effect of mushrooms on cytotoxic activity and cytokine production of intestinal lamina propria leukocytes does not necessarily depend on β -glucan contents

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Abstract

We evaluated the effects of seven mushroom extracts (*Grifola frondosa*, *Pholiota nameko*, *Panellus serotinus*, *Hypsizygos marmoreus*, *Pleurotus cornucopiae*, *Armillaria mellea*, and *Flammulina velutipes*) on cytotoxic activity and cytokine production of lamina propria leukocytes (LPLs) isolated from rat small (S) and large (L) intestinal mucosa. Boiling water extracts from seven species of mushrooms showed no direct cytotoxicity against the YAC-1 target cells. However, prominent increases of cytotoxicity were observed in S- and L-LPLs co-cultured with *P. serotinus* extract. Cytokine production (TNF α , IFN γ , IL-12 p70, and IL-4) of S- and L-LPLs was stimulated in response to *P. cornucopiae* extract. Mushroom extracts contribute to target cell adhesion and/or cytokine production in the effector cells. The promotion of cytotoxic activity in S- and L-LPLs was not necessarily related to β -glucan content of the mushroom.

Keywords: β -glucan / Cytotoxicity / Intestine / Lamina propria leukocyte / Rat

1 Introduction

Cultured immune cell lines, splenocytes, and mesenteric lymphocytes are widely used to investigate the direct influences of dietary factors on the immune system (Byeon *et al.*, 2009; Hoshi *et al.*, 2008; Lau *et al.*, 2004; Yoon *et al.*, 2003). The *in vivo* composition of immune cells differs among tissues (Ferguson & Engelhard, 2010); even within the intestine, lamina propria immune cell composition is very different from that of Peyer's patches (Lyscom & Brueton, 1982). Outside of Peyer's patches, immune cells in the intestinal mucosa are scattered in the lamina propria and intraepithelial regions (Brandtzaeg *et al.*, 2008), and disruptions of intestinal mucosal homeostasis may evoke a variety of diseases (Newberry & Lorenz, 2005).

Immune cells in the intestinal mucosa help maintain homeostasis through processes such as epithelial proliferation (Mennechet *et al.*, 2002) and B-cell attraction (Fujihashi *et al.*, 1996) into the lamina propria. Villous M cells and dendritic cells in non-Peyer's-patch subepithelial regions take up luminal antigens and present antigens to T cells (Jang *et al.*, 2004; Niess *et al.*, 2005; Rescigno *et al.*, 2001; Yamamoto *et al.*, 2000). Intestinal mucosal cells can thus recognize, monitor luminal antigens, and comprise the first line of immune cells encountered by dietary factors. Given the difficulty of isolating intestinal immune cells, however, we have a limited understanding regarding the influence of dietary factors on intestinal immune cell function.

Many species of edible mushrooms have historically been used in cuisine and alternative medicine for hundreds of years in Asia. Purified extracts of mushrooms have exhibited immunomodulatory activities (Borchers *et al.*, 2008), and most studies on the immunomodulatory effects of mushrooms or β -glucans on intestinal immune cells have been conducted in mice (Shen *et al.*, 2007; Tsukada *et al.*, 2003; Volman *et al.*, 2010). However,

more cells can be isolated from the intestinal mucosa of rats than from mice, presenting an advantage for such investigations. Our previous study (Lee *et al.*, 2009a) established a reliable method for isolating immune cells from the small (S) and large (L) intestinal mucosa of rats. The present study evaluated the direct influences of crude mushroom extracts, as dietary factors, on cytotoxic activity in intestinal mucosal immune cells.

2 Materials and methods

2.1 Rats

DA/Slc male rats (8 weeks old; Japan SLC, Hamamatsu, Japan) were individually housed throughout the experimental period in a temperature-controlled room under a 12-h photoperiod (lights on, 08:00–20:00 h) with free access to drinking water and a purified diet. The diet contained 60.25 g/kg sucrose, 25 g/kg casein, 5 g/kg microcrystalline cellulose (PH-102; Asahi Kasei Co., Tokyo, Japan), 5 g/kg soybean oil, 0.25 g/kg choline bitartrate, 3.5 g/kg AIN-93G mineral mixture (Reeves *et al.*, 1993), and 1 g/kg AIN-93 vitamin mixture (Reeves *et al.*, 1993). This study was approved by the Animal Care and Use Committee of Hokkaido University, and the animals were maintained under the university's guidelines for the care and use of laboratory animals.

2.2 Preparation of mushroom extracts

The Hokkaido Forest Products Research Institute (Asahikawa, Japan) provided seven mushroom species (*Grifola frondosa*, *Pholiota nameko*, *Panellus serotinus*, *Hypsizygus marmoreus*, *Pleurotus cornucopiae*, *Armillaria mellea*, and *Flammulina velutipes*). The

mushrooms were boiled at 90°C for 2 h, and the water-soluble supernatant was then lyophilized. The extracts were dissolved in complete RPMI-1640 medium (pH 7.4) for 3 h with agitation at room temperature. This medium contained 10% (v/v) heat-inactivated fetal bovine serum, 25 mM HEPES, 23.8 mM NaHCO₃, 0.17 mM streptomycin sulfate, 0.01 mM penicillin G potassium, and 50 μM 2-mercaptoethanol. Filtration was conducted with 5.0, 0.45 μm (Advantec®; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and 0.22 μm (MN sterilizer PES; Macherey-Nagel GmbH & Co. KG, Düren, Germany) filters, and samples of the mushroom extracts were then collected. The resulting 46 samples were stored in a cold room until use.

2.3 Determination of β-glucan concentration in mushroom extracts

The percentage of β-glucan in the water-soluble supernatants was quantitatively determined with a β-glucan assay kit for mushrooms and yeast (Megazyme International Ireland Ltd., Wicklow, UK) according to the manufacturer's instruction. Briefly, for the determination of total (α- and β-) glucan content, boiling water extracts (n = 3–6) isolated from the mushrooms were hydrolyzed with 1.3 M HCl at 100°C for 2 h. After pH was neutralized with 2 M KOH, the aliquot was digested with exo-1,3-β glucanase (20 U/ml) plus β-glucosidase (4 U/ml) in 200 mM of sodium acetate buffer (pH 5.0). The hydrolysates were incubated with a mixture of glucose oxidase and peroxidase at 40°C for 1 h. The absorbance of the solution was measured at 510 nm. For determining the α-glucan (phytoglycogen and starch) content, the extracts were dissolved in 2 M KOH, hydrolyzed, and added 1.2 M of sodium acetate buffer (pH 3.8). Amyloglucosidase (1630 U/ml) and invertase (500 U/ml) were added to the solution and incubated at 40°C for 30 min. The aliquot was incubated with a mixture of glucose oxidase and peroxidase at 40°C for 20 min. The absorbance of the solution was measured at

510 nm. The concentration of β -glucan was determined by subtracting α -glucan from total glucan content (Fig. 1A).

2.4 Preparation of leukocytes isolated from small and large intestinal mucosa

After an acclimation period, the rats were decapitated (10:00–11:00 h) under diethyl ether (Kanto Chemical Co., Inc., Tokyo, Japan) anesthesia and the S and L intestines were removed. Leukocytes were collected from the lamina propria of each intestinal tissue, as previously described (Lee *et al.*, 2009a). The isolated lamina propria leukocytes (LPLs, 1.0×10^6 cells) were washed twice in phosphate-buffered saline (PBS) and incubated for 30 min at 4°C in darkness with FITC-anti-CD3 mAb (clone IF4; AbD Serotec, Morphosys UK Ltd., Oxford, UK) and phycoerythrin-anti-CD161 mAb (clone 10/78; AbD Serotec). The stained cell fractions were then immediately applied to FACSCalibur (Becton, Dickson and Company, Franklin Lakes, NJ, USA), and the data (300,000 total events) were analyzed using FlowJo (version 7.2.5; TreeStar Inc., Ashland, OR, USA). Populations of CD3⁺ and CD161⁺ cells were confirmed in every isolated leukocyte fraction from the S and L intestinal mucosa. A representative profile is shown in Fig. 1B.

2.5 Cytotoxic activities of LPLs

LPL cytotoxic activity was measured with a LIVE/DEAD® cell-mediated cytotoxicity kit (Invitrogen Co., Carlsbad, CA, USA), as previously described (Lee *et al.*, 2009a). In brief, cultured NK-sensitive YAC-1 virus-induced mouse T-cell lymphoma cells (as target cells) were washed with PBS, and stained with 3,3'-dioctadecyloxycarbocyanine (DiOC; 5.5 mM) in PBS for 20 min at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The stained

YAC-1 target cells after incubation period were washed with PBS and mixed with isolated LPLs (as effector cells) with or without mushroom extract in 1 ml of the complete medium at an effector:target cell ratio of 50:1. The cell suspensions were then incubated with propidium iodide (PI; 3.75 mM) for 2 h in a humidified atmosphere of 95% air and 5% CO₂. The data (300,000 total events) were obtained with FACSCalibur (Becton, Dickson and Company), and the percentage of PI-stained dead YAC-1 cells was evaluated using FlowJo (TreeStar Inc.).

2.6 Effector cell cytokine production in response to mushroom extracts

The following ELISA kits were used in this study: IFN γ (Komabiotech, Seoul, Korea), ultrasensitive TNF α (BioSource, Camarillo, CA, USA), IL-1 β (BioSource), IL-2 (BioSource), IL-4 (BioSource), and IL-12 p70 (BioSource). Cytokine concentrations in the culture supernatant were evaluated according to the manufacturers' instructions. Cytokine production was determined by ELISA at 24 h after co-culture of effector cells and YAC-1 target cells with or without mushroom extract. A separate experiment followed the same procedure without the addition of effector cells. Effector cell cytokine production was calculated by subtracting the cytokine concentration in the co-culture from the average cytokine concentration produced in the culture without effector cells.

2.7 Adhesion ability of YAC-1 cells

After dissolution in complete RPMI-1640 medium (0.1, 1, or 10 mg/ml), samples of each mushroom extract (100 μ l) were placed in a 96-well plate (Corning Inc., Corning, NY, USA) and incubated for 1 h. The medium was then removed, and YAC-1 cells (5×10^4) from each

sample were incubated for 2 h in the extract-coated wells. The cell suspension was removed and the wells were washed once with the complete RPMI-1640 medium. A cell counting kit-8 (Dojindo, Kumamoto, Japan) was used to determine the number of cells remaining in the wells. The YAC-1 cell adhesion ability of each mushroom extract was defined as the proportion of remaining cells to loaded cells.

2.8 Direct cytotoxicity of β -glucan against YAC-1 cells

The direct cytotoxicity of β -glucan was measured with commercial β -glucan isolated from *Saccharomyces cerevisiae* (95% purity; Calbiochem, Darmstadt, Germany). YAC-1 cells were stained with DiOC as described above. The stained cells were exposed to the β -glucan, which had been dissolved in complete RPMI-1640 medium (0.02, 0.2, or 2 mg/ml). The cell suspension was then incubated with PI for 2 h in a humidified atmosphere of 95% air and 5% CO₂. The data (300,000 total events) were obtained with FACSCalibur (Becton, Dickson and Company), and the percentage of dead YAC-1 cells was evaluated using FlowJo (TreeStar Inc.).

2.9 Statistical analysis

Values are expressed as the mean \pm SD (Fig. 1A) and mean \pm SEM (Figs. 2–4). Student's *t*-test and Dunnett's test were used to compare two groups and multiple groups, respectively. Differences were considered statistically significant if the probability was less than 0.05. Statistical analyses were conducted using the JMP 5.0 software package (SAS Institute Inc., Cary, NC, USA).

3 Results

The mushroom extracts did not directly influence YAC-1 viability in the 2-h culture in this study (data not shown). Thus, the cytotoxic activity of isolated LPLs from S and L intestinal mucosa was evaluated in the presence (0.1, 1, or 10 mg/ml) or absence of mushroom extracts (Fig. 2). YAC-1 cytotoxicity in S- and L-LPLs without mushroom extracts was identical (S-LPLs, $22.2 \pm 2.2\%$; L-LPLs, $23.6 \pm 2.1\%$). The boiling water mushroom extracts promoted cytotoxic activity of S- and/or L-LPLs, depending on the extract source. *P. serotinus* (10 mg/ml) was observed to significantly promote YAC-1 cytotoxicity in both S- and L-LPLs. The effects of the mushroom extracts on cytotoxic activity were not always identical. For example, *H. marmoreus* and *P. cornucopiae* extracts promoted the cytotoxic activity of S-LPLs, but not L-LPLs. In contrast, *G. frondosa*, *P. nameko*, *A. mellea*, and *F. velutipes* extracts promoted the cytotoxic activity of L-LPLs, but not S-LPLs.

Our evaluation of the mushroom extracts' YAC-1-cell adhesion ability (Fig. 3) yielded values of approximately 16% YAC-1 cells without extracts in the loaded cell samples. While all mushroom extract types promoted the adhesion of YAC-1 cells on the culture plates at concentrations of 10 mg/ml, adhesion abilities at lower concentrations differed among extracts. *H. marmoreus* and *A. mellea* extracts showed adhesion ability at a concentration of 0.1 mg/ml. The highest adhesion abilities were observed in the *A. mellea* and *G. frondosa* extracts, which showed adhesion in 60% and 48% of loaded YAC-1 cells, respectively.

We also measured cytokine production in response to the mushroom extracts in S- and L-LPLs co-cultured with YAC-1 cells (Fig. 4). *P. cornucopiae* extracts almost always stimulated cytokine production in S- and L-LPL except for IL-2 in S-LPLs and IL-1 β in L-LPLs. *H. marmoreus*, *A. mellea*, and *F. velutipes* extracts did not promote any cytokine production in S-LPLs in this study. The production of IFN γ was stimulated in L-LPLs in response to *G.*

frondosa, *P. nameko*, *P. serotinus*, *P. cornucopiae*, *A. mellea*, and *F. velutipes* extracts. In addition, the production of IL-2 was stimulated in L-LPLs in response to *P. nameko*, *P. cornucopiae* extracts, but not in S-LPLs. L-LPLs appeared to exhibit a more sensitive cytokine production response to the mushroom extracts than S-LPLs.

This study measured the β -glucan concentrations of the seven mushroom extracts (Fig. 1A). The highest β -glucan contents were found in *G. frondosa* and *P. nameko* extracts, while the *P. cornucopiae*, *A. mellea*, and *F. velutipes* extracts contained less than 10% β -glucan. The *P. serotinus* and *H. marmoreus* extracts contained marginal β -glucan concentrations. The average concentration of β -glucan in the mushroom extracts analyzed here was about 17%. We also evaluated the direct cytotoxic activity of commercial β -glucan (0.02, 0.2, or 2 mg/ml) against YAC-1 cells isolated from *S. cerevisiae* (Fig. 5). The concentrations used in this experiment were determined by the average β -glucan content of the extracts. There was no direct cytotoxic activity of seven mushroom extracts against YAC-1 target cells. However commercial β -glucan from *S. cerevisiae* significantly reduced the viability of YAC-1 cells even during a 2-h culture.

4 Discussion

We previously investigated the influence of dietary factors on mucosal homeostasis especially via mucosal immune cells, and elucidated that fermentable dietary fibers regulate distribution of CD8 α^+ intraepithelial lymphocytes (IELs) in the large intestine by using a histochemical technique (Ishizuka & Tanaka, 2002; Ishizuka *et al.*, 2004). Moreover, we demonstrated that CD8 α^+ IELs contributes to expression of CCL28, a chemokine for IgA $^+$ B cells, in the villus epithelia (Lee *et al.*, 2009b). In general, many researchers use splenocytes or mesenteric

lymphocytes to determine the influence of dietary components on immune cell functions. However, the composition of the cells in spleen and mesenteric lymph nodes is very different from that in intestinal mucosa (Lee *et al.*, 2009a). The responses of splenocytes or mesenteric lymphocytes to dietary factors might be different from that of mucosal lymphocytes. Thus, we focus on intestinal mucosal immune cells to determine the direct influences of dietary factors on the immune system. This study evaluated the ability of boiling water extracts from seven mushroom species to activate the cytotoxic activity of mucosal immune cells. As expected, we found that these extracts promoted cytotoxic activity in S- and L-LPLs, but we also observed differences among the extracts. *P. serotinus* extracts promoted cytotoxic activity of both S- and L-LPLs, while other extracts significantly influenced only S- or only L-LPLs (Fig. 2).

We observed no direct cytotoxicity of the mushroom extracts against YAC-1 cells without effector cell co-culturing. The mushroom extracts analyzed in this study promoted YAC-1 cytotoxicity by activating S- or L-LPLs. The effector cells were co-cultured with target cells in the presence of mushroom extracts for only 2 h in this study, which may have not been sufficient time to promote cytotoxic activity via cytokine productions from effector cells. We speculated that such mushroom extracts promote adhesion to target cells or activate exocytosis of cytotoxic granules in the effector cells.

Increase in adhesion ability of YAC-1 was observed in the extract sources of *G. frondosa*, *H. marmoreus*, and *A. mellea* (Fig. 3). All mushroom extracts analyzed in this study significantly increased cell adhesion at concentrations of 10 mg/ml, demonstrating their role in YAC-1 cell adhesion. No data on the YAC-1 viabilities were obtained at the 10-mg/ml concentrations of *G. frondosa* and *A. mellea* due to cell aggregation. Additionally, a 1-mg/ml concentration of these extracts promoted cytotoxicity in L-LPLs (but not in S-LPLs). Taken together, these results indicate that the cell adhesion ability of mushroom extracts may

promote cytotoxic activity in L-LPLs.

Some studies have shown that β -glucan isolated from mushroom (Shen *et al.*, 2007), seaweed (Vetvicka *et al.*, 2007), or *Saccharomyces cerevisiae* (Li *et al.*, 2007; Yoon *et al.*, 2008) influences the cytotoxic activity of immune cells, but β -glucan is simply showing structure of polysaccharides and contains a variety of molecules. Thus, mushrooms might have their own β -glucan. In this study, we investigated whether boiling water extracts from some types of mushrooms possess immunomodulatory effect on isolated mucosal immune cells. We also measured the β -glucan content of each extract analyzed in this study. The *G. frondosa* and *P. nameko* extracts had β -glucan contents of approximately 30 g/100 g. In contrast, the *P. cornucopiae*, *A. mellea*, and *F. velutipes* extracts had β -glucan contents of less than 10 g/100 g. Our analysis of the direct influence of commercial β -glucan on the viability of YAC-1 cells found that this β -glucan induced cell death without effector immune cells. These results demonstrate that the mushroom extracts' promotion of cytotoxicity through the activation of effector immune cells was not necessarily related to β -glucan content. It may instead be due to the structure of β -glucan, or another component of the extracts may have promoted cytotoxic activity. The ceramide component of some mushrooms has been reported to activate NKT cells (Im *et al.*, 2009; Okamoto *et al.*, 2005; Sharif *et al.*, 2001). However, the mushroom extracts used in this study may contain very little amount of ceramide due to hydrophobic property of the molecule.

The influence of mushroom extracts on cytokine production in suspensions of effector and target cell was also investigated. *P. cornucopiae* extracts promoted almost all cytokines tested in this study, and also increased S-LPL cytotoxicity. The extracts were co-cultured with effector and target cells for 2 h and 24 h for the cytotoxicity and cytokine production assays, respectively. Cell culture studies of cytokine production typically use 24- to 72-h periods (Masuda *et al.*, 2009; Pinto *et al.*, 2009; Ukawa *et al.*, 2007; Yu *et al.*, 2009). Longer co-

culturing periods are thus necessary for the detection of cytokine production than for that of cell cytotoxicity. Some mushroom extracts in this study, such as *P. nameko*, and *P. cornucopiae* increased the production of TNF α , IFN γ , and IL-2. These cytokines can influence intestinal mucosal homeostasis and immune surveillance (Chen *et al.*, 2009; Francoeur *et al.*, 2004; Gurbindo *et al.*, 1993; Robinson *et al.*, 2009; Stallmach *et al.*, 1999), and *in vivo* studies have found that the ingestion of mushroom extracts stimulates cytokine production.

We used boiling water extracts because mushrooms are usually cooked with boiling water before ingestion. Many types of mushrooms were used in this study. In case of an animal experiment, however, it is very difficult to determine the effect of a wide variety of extracts due to sample amount and duration of experiment. To determine physiological influence of dietary factors using animal experiment, it would take at least a couple of weeks. In this experiment, we isolated beyond 10^7 immune cells from intestinal mucosa per rats. Such alternative experiments contribute to reduce the number of animals to be used in an *in-vivo* study. Thus, *in-vitro* experiments such as those used in this study can be used as a first screening to select suitable mushroom extracts for subsequent animal experiment.

In conclusion, boiling water mushroom extracts promoted cytotoxic activity and cytokine production in mucosal immune cells. This promotion differed among extracts, suggesting that it may not be due solely to β -glucan content. This study also demonstrated that functional analyses using isolated intestinal mucosal immune cells can effectively evaluate the influences of dietary factors. Obviously, we need to do *in-vivo* experiment to understand precise influence of mushroom extracts on mucosal homeostasis. However, we can select some suitable extracts among the mushroom extracts used in this study for future *in-vivo* experiment.

Acknowledgments

This study was supported by funding from a joint project between the Hokkaido government and Hokkaido University.

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Figure captions

Figure 1. Concentration of β -glucan in mushroom extracts and proportions of CD3⁺ and CD161⁺ cells in mucosal leukocyte fractions isolated from small and large intestines of rats. (A) β -Glucan concentration in each mushroom extract was calculated by subtracting α -glucan from total glucan. Each value is expressed as the mean \pm SD (n = 3–9). (B) Lamina propria leukocytes (LPLs) collected from small and large intestines were stained with specific antibodies against CD3 (clone IF4; surface marker for T cells) and CD161 (clone 10/78; surface marker for natural killer cells). Each value is the representative percentage for a quadrant.

Figure 2. Cytotoxic activity against YAC-1 cells of leukocyte fractions isolated from the intestinal mucosa of rats exposed to mushroom extracts for 2 h. YAC-1 cytotoxicity is shown as the proportion of dead cells among YAC-1 target cells in the presence of effector cells, corrected for spontaneous target cell death in the absence of effector cells. The mushroom extracts alone demonstrated no significant influence on YAC-1 cell viability (data not shown). Each value is expressed as the mean \pm SEM (n = 3–9).

* vs. the value without mushroom extracts in S- and L-LPL groups ($P < 0.05$, by Dunnett's test).

S- vs. L-LPL groups ($P < 0.05$ by Student's *t*-test).

Figure 3. Adhesion ability of mushroom extracts for YAC-1 cells. Mushroom extracts (0.1, 1, or 10 mg/ml) in the complete RPMI 1640 medium were added to the wells of a culture plate. After removing the medium, 5×10^4 YAC-1 cells/well were added and incubated for 2 h. The wells were washed with complete RPMI 1640 medium and the remaining YAC-1 cells were

counted. The percentage of remaining cells was taken as the YAC-1 cell adhesion ability. Each value is expressed as the mean \pm SEM (n = 3–9).

* vs. the value without mushroom extracts ($P < 0.05$, by Dunnett's test).

Figure 4. Cytokine production by LPLs isolated from rat small and large intestines following *in vitro* stimulation with mushroom extracts. Each cell fraction isolated from the lamina propria of small or large intestine was incubated for 24 h with YAC-1 target cells in the presence or absence of water-soluble mushroom extracts (10 mg/ml). Cytokine production from effector cells was evaluated by subtracting the concentration of a cytokine in a co-culture containing effector cells, target cells, and mushroom extract from the average value of the cytokine concentration produced in a separate culture without effector cells. Values are expressed as the mean \pm SEM (n = 3).

* vs. the value without mushroom extract in S- and L-LPL groups ($P < 0.05$, by Dunnett's test).

S- vs. L-LPL groups ($P < 0.05$ by Student's *t*-test).

Figure 5. Direct cytotoxicity against YAC-1 cells. Cytotoxicity against YAC-1 cells of commercial β -glucan isolated from *Saccharomyces cerevisiae*. Each value is expressed as the mean \pm SEM (n = 4).

* vs. the value without β -glucan ($P < 0.05$, by Dunnett's test).

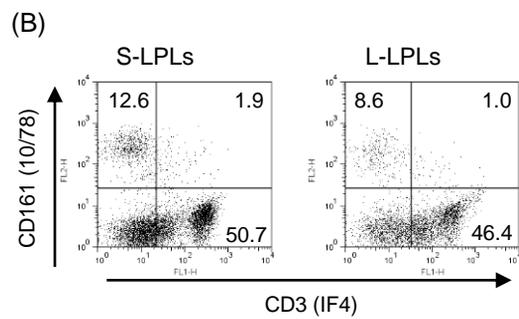
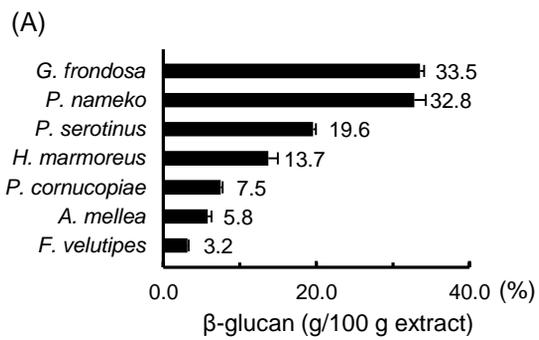


Fig. 1. LEE *et al.*

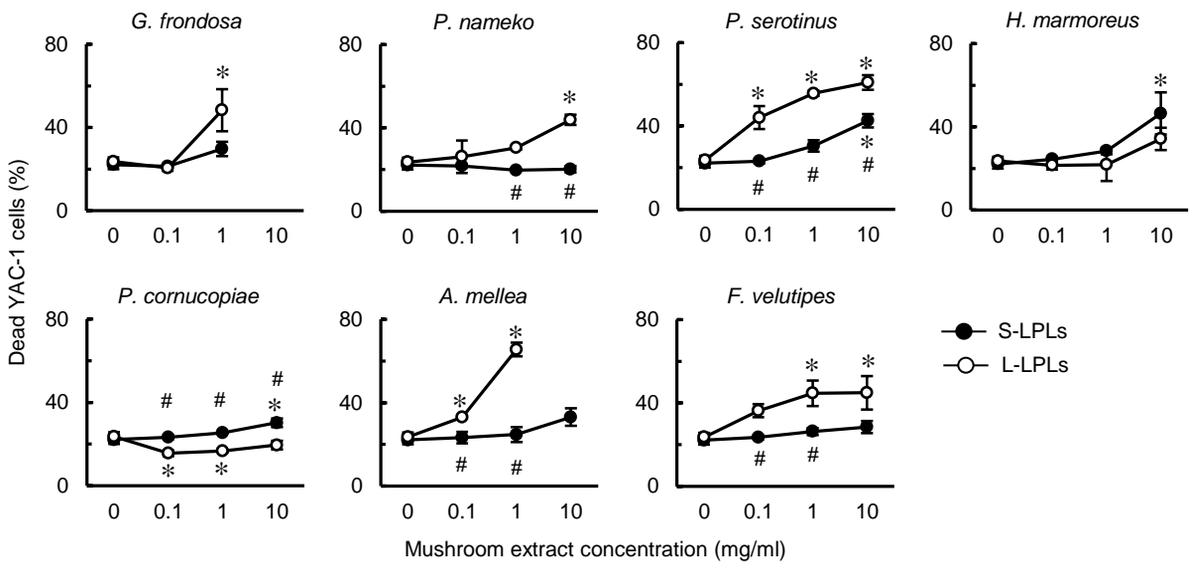


Fig. 2. LEE *et al.*

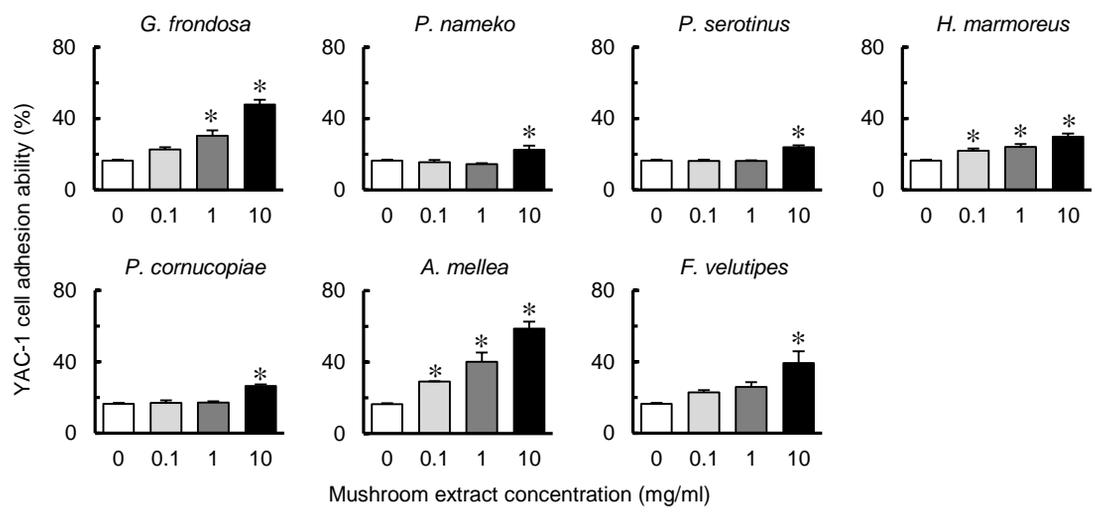


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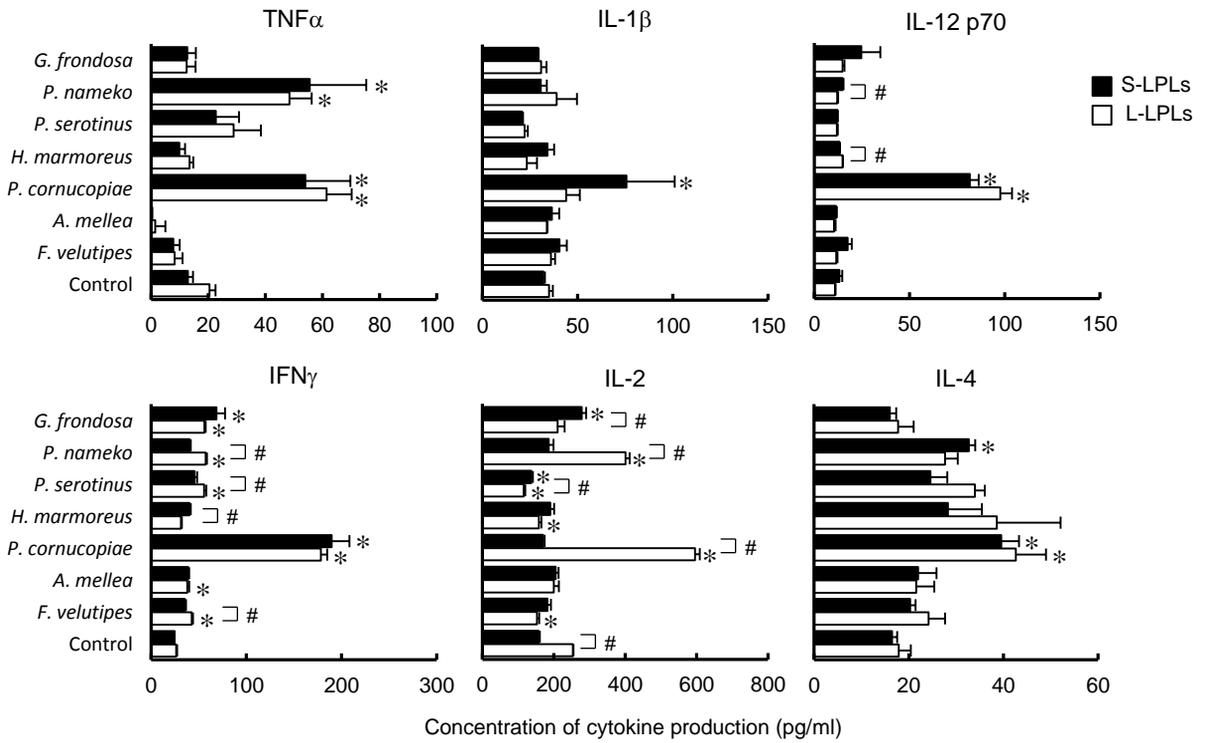


Fig. 4. LEE *et al.*

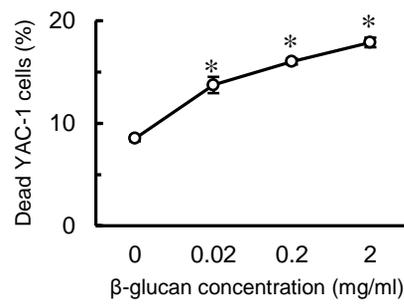


Fig. 5. LEE *et al.*