Food & Function

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Journal:	*Food & Function*
Manuscript ID	FO-ART-07-2019-001707.R1
Article Type:	Paper
Date Submitted by the Author:	29-Oct-2019
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Macrophage activation by edible mushrooms is due to the collaborative interaction of toll-like receptors agonists and dectin-1b activating beta glucans derived from colonizing microorganisms

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Abstract

Research supports the theory that the microbiome of plants and mushrooms produce potent activators of pathogen recognition receptors which are principal contributors to the stimulation of macrophages. We have previously reported that the *in vitro* macrophage stimulatory activity of water-soluble extracts from 13 different types of edible mushrooms is predominantly due to bacterial components originating from the naturally occurring bacterial communities within these materials. The purpose of the current study was to further investigate the bacterial-dependent activity of the water-soluble extracts and assess whether these 13 types of mushrooms contain water-insoluble beta glucans that activate the dectin-1b signaling pathway. Activity of the water-soluble extracts was predominantly due to Toll-like receptor 2 (TLR2) and TLR4 agonists. For dectin-1b-dependent activity (indicative of water-insoluble beta glucans), culinary mushrooms (*Agaricus bisporus* varieties) were essentially inactive, whereas most of the medicinal mushrooms (*Lentinula edodes, Grifola frondosa, Hypsizygus marmoreus* varieties*, Flammulina velutipes*) exhibited potent activation. *A. bisporus* samples with no detectable dectin-1b-dependent activity had yeast colony forming units that were 687 times lower than *L. edodes* exhibiting high activity, indicating that the active insoluble beta glucans are derived from colonizing yeast. In addition, co-stimulation of macrophages with the TLR agonists and insoluble beta glucan was found to result in a synergistic enhancement of *in vitro* cytokine production. Taken together, these findings indicate that the *in vitro* macrophage activating potential of edible mushrooms is due to the collaborative interaction of water-soluble TLR agonists (derived from colonizing bacteria) and water-insoluble beta glucans (derived from colonizing yeast).

Keywords

Fungi; mushroom; macrophage activation; beta glucan; dectin-1b; yeast.

Abbreviations

CFU, colony forming units; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; LAL, limulus amebocyte lysate; LPS, Lipopolysaccharide; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha; WGP, whole glucan particles.

Introduction

Mushrooms have historically been used both as a food source and for medicinal purposes such as enhancing immune function. Research indicates that both water-soluble and water-insoluble beta glucans contribute to the *in vitro* macrophage stimulatory activity exhibited by edible mushrooms and that these high molecular weight components are synthesized by the mushrooms.[1] However, the contribution of beta glucans to the overall immune-enhancing activity of mushrooms needs to be re-evaluated since evidence from both our research[2,3] and the literature[4] indicates that purified macromolecules isolated from complex matrices, such as natural products, often contain residual/trace components from other classes. These minor constituents may contribute to, interact with, or fully account for the biological activity of the isolated "pure" high molecular weight substance, and as a result, it can be difficult to correctly identify the main active compound(s). It is therefore possible that a portion of the macrophage stimulatory activity attributed to "purified" mushroom-derived beta glucans reported in the literature is due to other macromolecules present in these preparations at low levels.

In our previous research we investigated 13 types of edible mushrooms: 6 culinary (crimini, white button, morel, lobster, chanterelle, portobello) and 7 medicinal (maitake, shiitake, oyster, bunashimeji, bunapi, woodear, enoki).[5] Studies have demonstrated that these 7 medicinal mushrooms as well as *Agaricus bisporus* (white button, protobello, crimini) exhibit immune-enhancing properties.[6-12] We evaluated water-soluble extracts from the 13 types of mushrooms and found that cell wall components from bacteria colonizing these materials are principal contributors to their innate immune-enhancing activity.[5] Proteobacteria were the dominant bacterial phylum and *Pseudomonas* was the most abundant genus detected in the majority of the mushroom samples. Macrophage activation (TNF-α production) of extracts was significantly correlated with total bacterial load and content of LPS. Treatment of extracts with NaOH resulted in a 97-100% reduction in macrophage activation, providing evidence that the detected activity was due to bacterial components. Bacterial cell wall components such as LPS, Braun-type lipoproteins, and lipoteichoic acid are inactivated by base-dependent removal of ester- and amide-linked fatty acids. Based on these findings, our overall theory is that components derived from the naturally occurring microbial communities (microbiome) within

mushrooms are responsible for the innate immune-enhancing activity of these natural products.

One limitation of testing soluble extracts is that the activity contributed by insoluble components is not evaluated. Therefore, the purpose of the current study was to build on our previous research and evaluate the activity exhibited by insoluble beta glucans within the 13 different types of edible mushrooms. Insoluble beta glucans activate the dectin-1b signaling pathway[13] and oral administration these compounds produce immune-enhancing effects in animal models and human trails.[14] Since insoluble beta glucan is a cell wall component of fungal organisms, preliminary experiments were also conducted to assess whether these substances were derived from the mushroom or from colonizing yeast and/or molds. Average yeast and mold populations within mushroom tissue are reported to range between about 63-630,000 CFU/g and 158-50,000 CFU/g, respectively.[15]

Materials and Methods

Mushroom material

Twenty-six mushroom samples were obtained from local supermarkets (Oxford, MS and Memphis, TN) and included the following 13 types: white button (*Agaricus bisporus*), crimini (*Agaricus bisporus*), portobello (*Agaricus bisporus*), woodear (*Auricularia auricula-judae*), chanterelle (*Cantharellus cibarius*), morel (*Morchella esculenta*), lobster (*Hypomyces lactifluorum*), bunapi (*Hypsizygus marmoreus*), bunashimeji (*Hypsizygus marmoreus*), enoki (*Flammulina velutipes*), oyster (*Pleurotus ostreatus*), shiitake (*Lentinula edodes*), maitake (*Grifola frondosa*). Additional shiitake mushroom samples were obtained from various online suppliers throughout North America and, together with locally obtained material, comprised a total of 12 different batches: company A and C (fresh, two different batches from Monterey Mushrooms), company B (fresh, Whole Foods Market), company D (dried, TastePadThai), company E (dried, organic, and micronized from Purica), company F (dried organic from Mycological Natural Products), company G (dried, Murray International Trading), company H (dried, Dynasty), company I (dried, Swanson), company J (dried organic, Aloha Medicinals),

company K (dried artisan grown, Northwest Wild Foods), and company L (dried organic, Mountain Rose Herbs).

To mimic the culinary use of mushrooms, fresh mushroom samples were rinsed with tap water according to the label instructions except for oyster mushrooms (label indicated not to wash prior to use).

Sample preparation for evaluation of TLR2, TLR4, dectin-1b and dectin-1a-dependent activities

Each sample was freeze-dried, ground into a fine powder and stored at -20°C until analysis. Ground mushroom material (30-50 mg) was extracted four times with 95% ethanol (1.0 mL fresh solvent added and incubated at 75°C for 45-60 minutes for each extraction) to remove non-polar substances that may be inhibitors of macrophage activation. Ethanol extracted mushroom material was then dried overnight at 50–55°C.

Crude extracts were prepared for evaluation of TLR2 and TLR4-dependent activation by further extracting the dried ethanol extracted mushroom material with 0.5 mL of water containing 4% SDS at 98°C for 1 hour. SDS was then removed using SDS-out reagent (Thermo Fisher Scientific) in the presence of 1% octylglucoside. For assessment of collaborative interactions, shiitake mushroom extract containing TLR2 and TLR4 activators was prepared by further extracting the dried ethanol extracted material with 0.75 mls of water containing 4% SDS at 98°C for 1 hour, followed by removal of SDS using SDS-out reagent only.

Samples were prepared for evaluation of dectin-1b-dependent activation by subjecting the material to polytroning to create a fine suspension of insoluble particles. In brief, the ethanol-extracted mushroom material was suspended in DMEM medium (high D-glucose, 4.5g/L) containing antibiotics (penicillin at 50 units/ml and streptomycin at 50µg/ml) at a concentration of 10mg of mushroom material/ml. A fine suspension of particles was created by polytroning each sample at 30,000 RPM for 5 – 10 minutes using a Power Gen 125 homogenizer. Samples were then centrifuged (14,000 RPM for 5 minutes) to remove supernatant that could contain compounds such as water-soluble beta glucans that bind to the dectin-1b receptor and block activation of this signaling pathway. For evaluation of activity, centrifuged pellets were

resuspended in 1X phosphate buffered saline by vortexing and sonication. For evaluation of collaborative interactions, the shiitake ethanol-extracted material was further processed to remove water soluble components including TLR2 and TLR4 activators. This was accomplished by performing six extractions with 4% SDS (1.0 mL solvent, 98°C, 30-60 minutes for each extraction) followed by two extractions with water (1.0 mL solvent, 98°C, 10 minutes for each extraction). Final solvent-extracted material was subjected to polytroning to create a fine suspension of particles as described above.

For evaluation of soluble dectin-1a-dependent activity, the dried ethanol extracted mushroom material was further extracted with 3 mls of Milli-Q water at 98°C for 1 hour. Following centrifugation, supernatant extract was removed and filtered through a 0.22 µm syringe filter to ensure complete removal of any raw material particles. Filtered extracts were then assessed for activity.

Reporter cell lines and macrophage activation assay

Selective detection of activation of the various pathogen recognition receptors (PRR) was accomplished using HEK-Blue™ hTLR2, HEK-Blue™ hTLR4, HEK-Blue™ hDectin-1a, HEK-Blue™ hDectin-1b and HEK-Blue™ Null1 cells (InvivoGen). These cell lines are engineered to stably co-express a PRR gene and a NF-kappa B inducible secreted embryonic alkaline phosphatase (SEAP) gene. Activation is detected by measurement of SEAP levels in the culture medium using the QUANTI-Blue™ reagent with measurement of optical density at 635nm. Assays were performed based on recommended InvivoGen protocols.

Determination of macrophage activation was performed as previously described.[5] In brief, TNF-α levels were measured in culture supernatants from RAW 264.7 cells (ATCC) incubated with crude extracts for 18–24 h. The level of TNF-α was determined using enzyme-linked immunosorbent assays (ELISA) (R&D Systems) following the manufacturer's protocol.

Positive controls were purchased from InvivoGen and included ultra pure *E. coli* LPS from *E. coli* 0111:B4 (TLR4 agonist), Pam3CSK4 (TLR2 agonist) and 1,3/1,6-ß-glucans from *S. cerevisiae* WGP dispersible (dectin-1b agonist) and WGP soluble (dectin-1a agonist).

Determination of yeast and mold colony forming units (CFUs)

White button mushrooms (Fresh Selections, Lot. 05 1314 2) and shiitake mushrooms (Private Selection, Lot. 05 1512 2) were purchased fresh from Kroger Co., Oxford MS USA. Fruiting bodies were rinsed extensively in distilled water and homogenized at 1g wet weight/5 mL ultrapure water (Milli-Q, Synergy UV system) using a Power Gen 125 homogenizer. Homogenates were filtered through a 149µm filter and CFUs for yeasts and molds determined using 3M™ Petrifilm™ Rapid Yeast and Mold Count Plates according to manufacturer's instructions.

Determination of total bacterial load and LPS content

Determination of LPS content using the limulus amebocyte lysate (LAL) assay and estimation of total bacterial load using a PCR-based quantification method were previously performed[5] and methods used were described therein.

Statistical analysis

Values for bacterial load, endotoxin units and EC_{25} values for TNF-α production were $\log_{10}$ transformed prior to regression since they spanned several orders of magnitude and were not normally distributed. Pairwise linear-regressions were performed in Microsoft Excel 2016 (version 16.16.7). Differences were determined using analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) test and pairwise t-tests.

Results

Water-soluble mushroom extracts exhibit variation in TLR2- and TLR4-dependent activity but do not substantially activate the dectin-1a signaling pathway

Extracts of the 13 types of mushroom samples were evaluated to determine the activity contributed by TLR2 agonists (bacterial lipoproteins and lipoteichoic acid) and TLR4 agonists (bacterial lipopolysaccharides). Extracts exhibited substantial variation in activation of both the TLR2 and TLR4 signaling pathways (Fig. 1A-B). As expected, no sample induced SEAP levels above control values when evaluated in the HEK-Blue™ null1 cells (Fig. 1C). Level of TLR2- and

TLR4-dependent activation was highly correlated with TNF-α production from RAW 264.7 macrophages ($R^2 = 0.89$, $p < 0.0001$, Fig. 1D and $R^2 = 0.96$, $p < 0.0001$, Fig. 1E, respectively).

To evaluate the activity contributed by water soluble beta glucans, hot water extracts were evaluated for activation of the dectin-1a signaling pathway (Fig. 2A). Even at very high concentrations (1000μg/ml), only minimal activation was detected for extracts from chanterelle and morel mushrooms. No detectable activation was observed for any mushroom samples tested at lower concentrations between 250μg/ml and 10pg/ml (data not shown) and no sample induced SEAP levels significantly above control values when tested in the HEK-Blue™ null1 cells (Fig. 2B).

Water-insoluble mushroom materials exhibit substantial variation in dectin-1b-dependent activity (particulate beta glucan) that correlates with the number of yeast CFUs

Fine suspensions of insoluble material were prepared from the 13 types of mushroom samples and evaluated for particulate beta glucan activity (activation of the dectin-1b signaling pathway). The *Agaricus bisporus* varieties (white button, crimini and portobello) and woodear exhibited essentially no activity (Fig. 3A). By comparison, the other mushrooms activated the dectin-1b pathway and were about 20 times more potent than the WGP dispersible beta glucan positive control (Fig. 3A). As expected, no sample induced SEAP levels above control values in the HEK-Blue™ null1 cells (Fig. 3B).

To evaluate activity variation within the same mushroom, batches of shiitake were obtained from various sources that included different cultivation conditions (organic and non-organic) and different origins (United States and various countries in Asia). Despite differences in cultivation and origin, 10 of the 12 batches evaluated exhibited similar potency for activation of dectin-1b (Fig. 4). The lack of activity exhibited by the shiitake obtained from company E (Lot. P00100, Purica) and company J (Lot. 200217S.S, Aloha Medicinals), compared to other mushroom sources ($p<0.0001$), may be due to cultivation conditions. Both companies cultivate mushrooms at a commercial scale in a controlled laboratory environment to minimize the presence of microbial organisms and to avoid cross-contamination. The certificate of analysis

for Lot. 200217S.S indicated that the material contained < 100 CFU/g of yeast and mold and contains 25.19% 1,3-1,6 beta glucan. No certificate of analysis was available for Lot. P00100.

Yeast and mold counts were determined in fresh mushroom samples exhibiting no detectable dectin-1b-dependent activity (white button) and compared to samples containing potent activity (shiitake). Yeast counts were over 600 times higher for the shiitake as compared to the white bottom (average of 6179 versus 9 CFU/g, respectively, Table 1). By contrast, shiitake mushrooms had 7.6 times lower mold counts than white button mushrooms (average of 82 versus 620, respectively, Table 1).

Interactions between bacterially-derived TLR agonists and water-insoluble beta glucan

There was a significant inverse correlation between dectin-1b-dependent activity (insoluble beta glucans) and number of bacterial cells within the 13 types of mushroom samples (Fig. 5A, $R^2 = 0.18$, $p = 0.03$). Similarly, dectin-1b-dependent activity was inversely correlated with content of the bacterial component LPS within the mushroom samples (Fig. 5B, $R^2 = 0.32$, $p = 0.003$).

In RAW 264.7 macrophages, purified water-insoluble beta glucan (WGP dispersible from *S. cerevisiae*) induces low production of TNF-α production at 100µg/ml (Fig. 6). However, in combination with either TLR4 agonist *E. coli* LPS or TLR2 agonist Pam3CSK4, WGP dispersible enhanced TNF-α production above levels achieved by incubating cells with the TLR agonists alone ($p<0.05$). Soluble beta glucan (WGP soluble from *S. cerevisiae*) exhibited no detectable activity alone and did not enhance cytokine production when tested in combination with either *E. coli* LPS or Pam3CSK4 (Fig. 6).

For assessment of collaboration interactions between microbial derived components within mushrooms, shiitake was further investigated by preparing a soluble extract containing only TLR agonists (TLR2 ($p<0.05$) and TLR4 ($p<0.001$) activity detected but no dectin-1b activity detected, Fig. 7A) and an insoluble extract containing only water-insoluble particulate matter (dectin-1b activity only, $p<0.001$, Fig. 7B). Both extracts exhibited a dose response dependent activation of TNF-α production in RAW 264.7 macrophages and in combination synergistically

enhanced cytokine production above levels that would constitute an additive interaction ($p<0.001$, Fig. 7C).

Discussion

A growing body of evidence from our lab[2, 16-18] and others[19-20] support the theory that the naturally occurring microbial communities within plants produce potent activators of pathogen recognition receptors that are principal contributors to the *in vitro* stimulation of macrophages. For example, we recently reported that *Echinacea purpurea* contains bacterial loads ranging from 10^6 to 10^8 bacterial cells per gram of dry material[17] and that the *in vitro* macrophage activation exhibited by extracts can be fully accounted for by the activities and prevalence of Proteobacteria members of the plant microbiome.[18] The current study expands this theory to also include edible mushrooms. Results indicate that the *in vitro* macrophage activating potential of edible mushrooms is due to water-soluble TLR agonists (derived from microbiome bacteria) and water-insoluble particulate beta glucans (derived from microbiome yeast).

The current studies provide additional evidence supporting our previous research[5] that bacterial-derived components are responsible for the *in vitro* macrophage activity detected in the soluble mushroom extracts. The *in vitro* macrophage stimulatory activity of the water-soluble mushroom extracts appears to be predominantly due to TLR2 and TLR4 agonists. Variation in TNF-α production exhibited by the extracts was highly correlated with both TLR2- and TLR4-dependent activity (Fig. 1D and 1E). R-squared values of these regressions indicate that these TLR agonists accounts for about 90% of the variation in macrophage stimulatory activity. It is likely that the TLR2 and TLR4 agonists are of bacterial origin since we previously reported[5] that extract macrophage stimulatory activity was also highly correlated with number of bacterial cells and endotoxin units within the mushroom tissue. The detection of potent TLR2 and TLR4-dependent activities indicates that the majority of the bacteria within the mushroom are Gram-negative since these microbes contain both LPS (TLR4 agonists) and Braun-type lipoproteins (TLR2 agonists). Evidence supporting this conclusion was provided in our previous publication where Proteobacteria was found to account for over 80% of recovered sequences.[5]

Water-soluble beta glucans (dectin-1a agonists) contribute minimal, if any, activity to the water-soluble mushroom extracts. No dectin-1a-dependent activity was detected in the extracts, except at extremely high concentrations (1000μg/ml) for chanterelle and morel mushrooms (Fig. 2A). Furthermore, our data indicates that the water-soluble beta glucans do not activate macrophages, as indicated by undetectable TNF-α production in RAW 264.7 cells exposed to WGP soluble beta glucan from *S. cerevisiae* (Fig. 6).

Activity due to insoluble/particulate beta glucans (dectin-1b agonists) varied dramatically between the different types of mushrooms (Fig. 3A). Of potential significance was the observation that culinary mushrooms (the *Agaricus bisporus* varieties white button, crimini and portobello) exhibited essentially no activity. By comparison, most of the medicinal mushrooms (bunapi, bunashimeji, enoki, oyster, shiitake and maitake) exhibited potent activation of the dectin-1b signaling pathway. It is possible that a high level of active particulate beta glucans is one characteristic that defines why some mushrooms have been traditionally selected for medicinal purposes such as immune enhancement.

The substantial variation in dectin-1b-dependent activation between the different types of mushrooms was an unexpected finding. Beta glucans are the most abundant cell wall polysaccharide in fungi and the ß-1,3 structure comprises between 65 and 90% of the total beta glucan content.[21] Therefore, it was surprising that some of the mushrooms (*Agaricus bisporus* varieties and woodear) contained no beta glucans that activated the dectin-1b pathway. One possible explanation for this data is that there may be unique structural features between different mushroom beta glucans. However, since beta glucans are also a cell wall component of yeast and mold, we generated the hypothesis that the difference in dectin-1b-dependent activity is due to variation in the number of yeast and/or mold cells colonizing these materials. The strongest evidence supporting our hypothesis is that yeast counts were over 600 times higher in shiitake mushrooms (exhibiting potent activation of the dectin-1b pathway) as compared to white button mushrooms (exhibiting no activity). The large difference in yeast CFUs between these mushrooms agrees with literature values (average of 158 CFU/g for white button and 7943 CFU/g for shitake, Table 1[15]). Mold counts were higher for white button as compared to shiitake (620 versus 82 CFU/g, Table 1), indicating that mold is probably not

responsible for the difference in dectin-1b-dependent activity between these mushrooms. One area of future research would be to determine whether the low yeast CFUs in *Agaricus bisporus* could be due to the presence of anti-fungal compounds derived either from the mushroom and/or the colonizing bacterial communities.

Analysis of different batches of shiitake mushrooms obtained from various sources provided additional support for our hypothesis. If the dectin-1b activating particulate beta glucans were derived from mushroom cell walls, then similar levels of activity should be observed for different batches of the same mushroom. Although 10 of the 12 samples analyzed exhibited similar potency for dectin-1b-dependent activity, 2 batches exhibited minimal or no activity (Fig. 4). The batches that lacked activity were sourced from 2 companies (Aloha Medicinals and Purica) that cultivate shiitake mushrooms in control laboratory environments to minimize the presence of microbial organisms and to prevent cross-contamination. The batch from Aloha Medicinals exhibited no detectable activity (company "J", Fig. 4) and had total yeast and mold counts less than 100 CFU/g. A certificate of analysis with microbial quality control parameters was not available from Purica. It is therefore possible that the low dectin-1b-dependent activity detected in these two mushroom samples was due to dramatically lower levels of colonizing microorganisms such as yeast.

Bacterial and fungal communities often coexist and can have complex interactions ranging from antagonistic to synergistic.[22] Within the 13 types of edible mushrooms, we observed significant inverse correlations between dectin-1b-dependent activity (insoluble beta glucans that are likely derived from colonizing yeast) and total bacterial load as well as content of LPS (Fig. 5). These results suggest that there is an antagonistic interaction between the bacterial and yeast communities with edible mushrooms. The low R-squared values (0.18 and 0.32, Fig. 5) does, however, indicate that the antagonistic interaction is weak or limited to specific microbial members.

In vitro studies[23] indicate that the combination of particulate beta glucan (a dectin-1 agonist) and a TLR2 or TLR4 agonist results in a synergistic enhancement of an immune response (detected as enhanced cytokine levels). Data from our experiments also demonstrate that maximally activating concentrations of *S. cerevisiae* particulate beta glucan (WGP

dispersible), in combination with Pam3CSK4 (TLR2 agonist) and ultra-pure LPS (TLR4 agonist), synergistically enhances TNF-α production from RAW 264.7 macrophages (Fig. 6). However, water-soluble beta glucan from *S. cerevisiae* (WGP soluble), neither activates macrophage independently nor does it enhance activation in combination with Pam3CSK4 and ultra-pure LPS (Fig. 6). As would be expected, additional experiments demonstrated a collaborative interaction between the water-soluble TLR agonists and insoluble dectin-1b activating beta glucans within shiitake mushroom (Fig. 7). These results suggest that the total *in vitro* macrophage activating potential of mushrooms is due to the collaborative interaction of insoluble particulate beta glucans with TLR2 and TLR4 agonists.

Conclusion

Microbial research on edible mushrooms has generally focused on organisms that are pathogenic and involved in food spoilage. However, the bacteria and yeast that assemble the mushroom microbiome likely also include members that could have a positive impact on human health. Probiotic research has demonstrated that consumption of specific types of yeast (e.g., *Saccharomyces boulardii*) and bacteria (e.g., *Lactobacillus rhamnosus*) enhance host immune health and provide resilience against infections.[24] It is likely that microbial-derived components are responsible for many of the immune-mediated effects of probiotics, since similar *in vivo* effects are observed with administration of both live and heat-killed bacteria (e.g., protection against viral infection[25, 26]). Similarly, research demonstrates that yeast-derived beta glucans exert health effects such as enhanced immune defense against infections.[27] Therefore, the medicinal properties that have been attributed to some mushrooms may be dependent on the TLR agonists and insoluble dectin-1b activating beta glucans derived from the microbial communities that colonize these food products.

Acknowledgements

This research was partly funded by Grant Number R01AT007042 from the National Center for Complementary and Integrative Health (NCCIH) and the Office of Dietary Supplements (ODS). The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the NCCIH, ODS or the National Institutes of Health. Additional funding of this research was also provided by the USDA, Agricultural Research Service Specific Cooperative Agreement No. 58-6060-6-015. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA is an equal opportunity employer.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

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Table 1. Yeast and mold colony forming units (CFUs) determined in fresh *Agaricus bisporus* (white button) and *Lentinula edodes* (shiitake) mushrooms.

Mushroom	**Sample**	**Yeast (CFU/g)**	**Molds (CFU/g)**
White button	1	15	90
	2	50	50
	3	0	0
	4	0	1,600
	5	0	1,700
	6	0	600
	7	0	300
	Average	**9**	**620**
Shiitake	8	3,250	75
	9	12,500	0
	10	20,000	500
	11	3,300	0
	12	1,200	0
	13	2,800	0
	14	200	0
	Average	**6179**	**82**
Literature Data*		**Yeast CUF/g (range)**	**Molds CFU/g (range)**
White button	n=20	158 (0-1000)	158 (0-398)
Shiitake	n=30	7943 (1259-63,096)	7943 (398-63,096)

*Venturini ME et al (2011), *Food Microbiology* 28: 1492-1498.

Figure legends

Figure 1. Mushroom macrophage stimulatory activity exhibited by water-soluble extracts is predominantly due to TLR agonists. Extracts from 13 types of mushrooms were evaluated in HEK-Blue™ hTLR2 cells for TLR2-dependent activity (A), HEK-Blue™ hTLR4 cells for TLR4-dependent activity (B), and control HEK-Blue™ null1 cells (C). Response ratio is defined as OD of sample/OD of negative control. Positive controls ultra pure *E. coli* LPS and Pam_3CSK_4 were each tested at 100ng/ml. TLR2- and TLR4-dependent activities exhibited by extracts of the 13 types of mushrooms (n=26) were plotted against TNF-α production from RAW 264.7 macrophages (D and E, respectively). EC_{25} values represent the concentration (μg/ml) of mushroom material required to induce cytokine production to levels 25% of those achieved by ultra pure *E. coli* LPS (100 ng/ml).

Figure 2. Mushrooms contain essentially no dectin-1a-dependent activity. Extracts from 13 types of mushrooms were evaluated in HEK-Blue™ hdectin-1a cells (A) and control HEK-Blue™ null1 cells (B). Response ratio is defined as OD of sample/OD of negative control. WGP soluble 1,3/1,6-β-glucan from *S. cerevisiae* was used as the positive control (0.04 μg/ml).

Figure 3. Variation in dectin-1b-dependent activity exhibited by 13 types of edible mushrooms. Mushroom preparations were evaluated in HEK-Blue™ hdectin-1b cells (A) and control HEK-Blue™ null1 cells (B). Response ratio is defined as OD of sample/OD of negative control. WGP dispersible 1,3/1,6-β-glucan from *S. cerevisiae* was used as the positive control (100 μg/ml).

Figure 4. Variation in dectin-1b-dependent activity in shiitake (*Lentinula edodes*) obtained for various sources (companies A – L). Samples were evaluated in HEK-Blue™ hdectin-1b cells and response ratio is defined as OD of sample/OD of negative control. *Samples from companies E and J had significantly lower activity compared to other mushroom sources, $p<0.0001$ (determined by two-way ANOVA followed by Tukey's HSD).

Figure 5. Correlations to bacterial load (A) and LPS content (B) with dectin-1b-dependent activity exhibited by 13 different types of edible mushrooms (n=26). Response ratio is defined as OD of sample/OD of negative control.

Figure 6. Collaborative interaction between insoluble yeast beta glucan and TLR agonists. RAW 264.7 macrophages were treated with dectin-1a agonist WGP soluble 1,3/1,6-β-glucan ("WGP sol", 100μg/ml), dectin-1b agonist WGP dispersible 1,3/1,6-β-glucan ("WGP dis", 100μg/ml), TLR4 agonist ultra pure *E. coli* LPS ("LPS", 100ng/ml), TLR2 agonist Pam_3CSK_4 ("Pam", 100ng/ml) and various combinations. "NC" refers to untreated cells. Treatment was 18 hours and TNF-α in culture supernatant was measured by ELISA. *Treatment with "LPS+WGP dis" and "Pam+WGP dis" significantly enhanced TNF-α production compared to "LPS" or "Pam", respectively, $p<0.05$ (determined by one-way ANOVA).

Figure 7. Collaborative interaction between TLR agonists and dectin-1b agonists within shiitake (*Lentinula edodes*). A soluble extract was prepared containing only TLR2 and TLR4 agonists (A) and an insoluble extract was prepared containing water-insoluble particulate matter exhibiting only dectin-1b dependent activity (B). Presence/absence of activity was determined using ANOVA followed by pairwise t-test. Selective detection of the pathogen recognition receptors was accomplished using HEK-Blue™ hdectin-1b cells, HEK-Blue™ hTLR2 cells, HEK-Blue™ hTLR4 cells, and control HEK-Blue™ null1 cells (A, B). RAW 264.7 macrophages were treated with the soluble (black squares) and insoluble extracts (black circles) and in combination (white circles) at the indicated concentrations (C). Theoretical values for an additive interaction between the soluble and insoluble extracts is indicated by the dashed line with "X" symbols. Macrophages were treated for 18 hours and TNF-α in culture supernatant was measured by ELISA. Treatment with "Soluble + Insoluble Exts" significantly enhanced TNF-α production compared to other treatments, including the "Theoretical additive value". *$p<0.05$, **$p<0.001$.

Figure 1

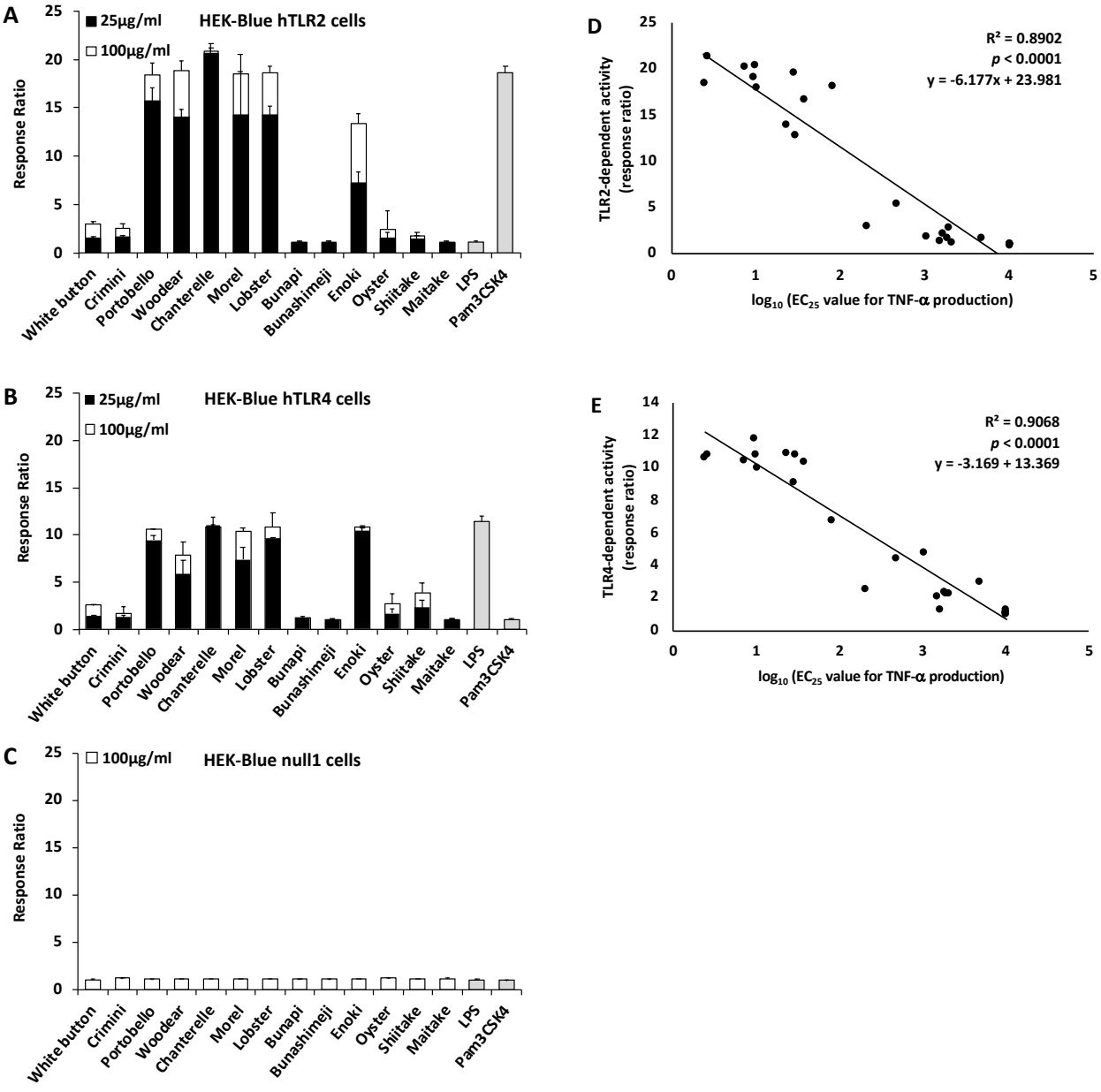
A
25μg/ml
100μg/ml
HEK-Blue hTLR2 cells
Response Ratio
White button
Crimini
Portobello
Woodear
Chanterelle
Morel
Lobster
Bunapi
Bunashimeji
Enoki
Oyster
Shiitake
Maitake
LPS
Pam3CSK4
B
25μg/ml
100μg/ml
HEK-Blue hTLR4 cells
Response Ratio
C
100μg/ml
HEK-Blue null1 cells
Response Ratio
D
R² = 0.8902
p < 0.0001
y = -6.177x + 23.981
TLR2-dependent activity (response ratio)
log10 (EC25 value for TNF-α production)
E
R² = 0.9068
p < 0.0001
y = -3.169 + 13.369
TLR4-dependent activity (response ratio)
log10 (EC25 value for TNF-α production)

Figure 2

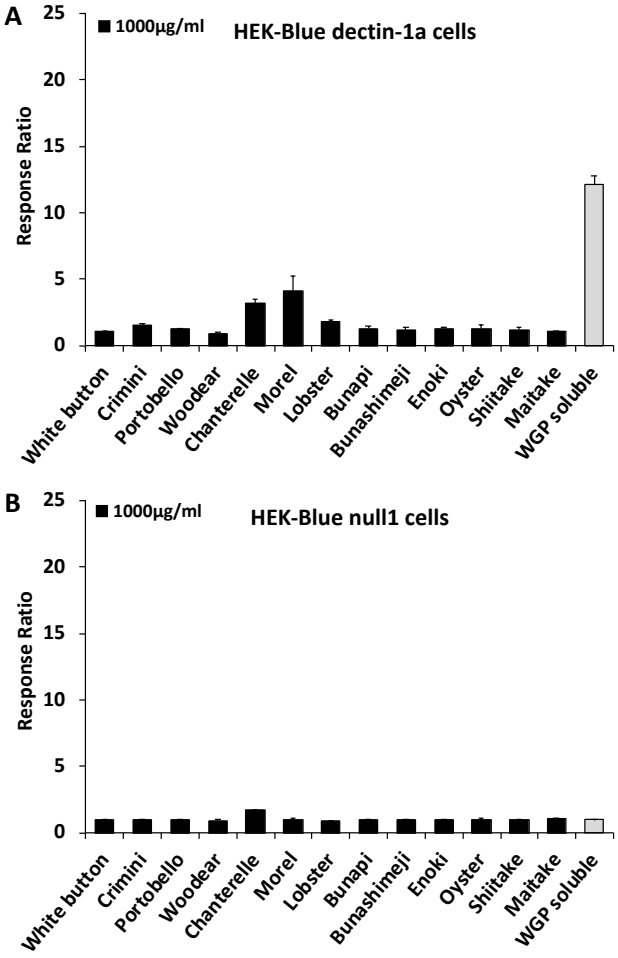
A
1000μg/ml
HEK-Blue dectin-1a cells
Response Ratio
25
20
15
10
5
0
White button
Crimini
Portobello
Woodear
Chanterelle
Morel
Lobster
Bunapi
Bunashimeji
Enoki
Oyster
Shiitake
Maitake
WGP soluble
B
1000μg/ml
HEK-Blue null1 cells
Response Ratio
25
20
15
10
5
0
White button
Crimini
Portobello
Woodear
Chanterelle
Morel
Lobster
Bunapi
Bunashimeji
Enoki
Oyster
Shiitake
Maitake
WGP soluble

Figure 3

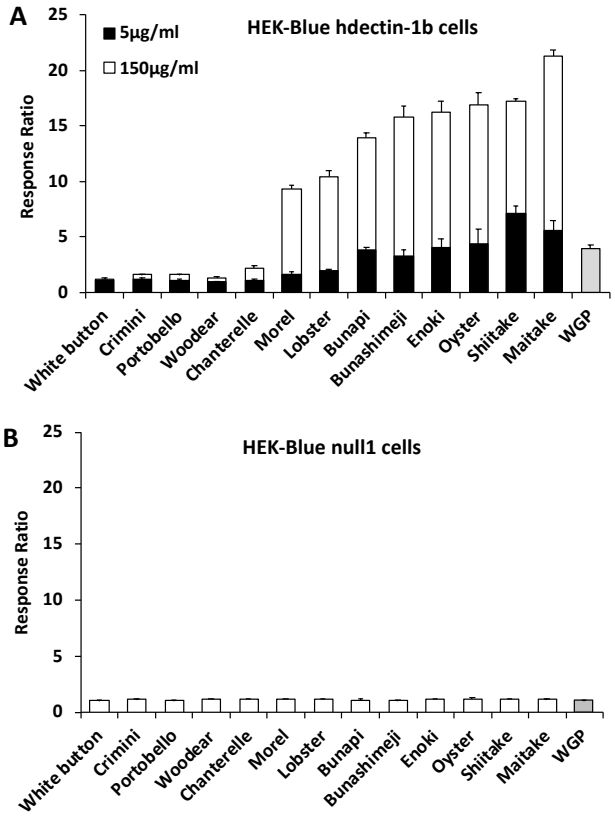
A
HEK-Blue hdectin-1b cells
5μg/ml
150μg/ml
Response Ratio
25
20
15
10
5
0
White button
Crimini
Portobello
Woodear
Chanterelle
Morel
Lobster
Bunapi
Bunashimeji
Enoki
Oyster
Shiitake
Maitake
WGP
B
HEK-Blue null1 cells
Response Ratio
25
20
15
10
5
0
White button
Crimini
Portobello
Woodear
Chanterelle
Morel
Lobster
Bunapi
Bunashimeji
Enoki
Oyster
Shiitake
Maitake
WGP

Figure 4

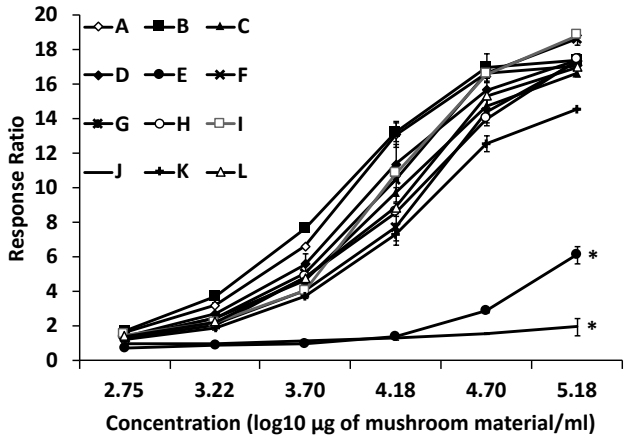
A
B
C
D
E
F
G
H
I
J
K
L
Response Ratio
0
2
4
6
8
10
12
14
16
18
20
2.75
3.22
3.70
4.18
4.70
5.18
Concentration (log10 µg of mushroom material/ml)
*
*

Figure 5

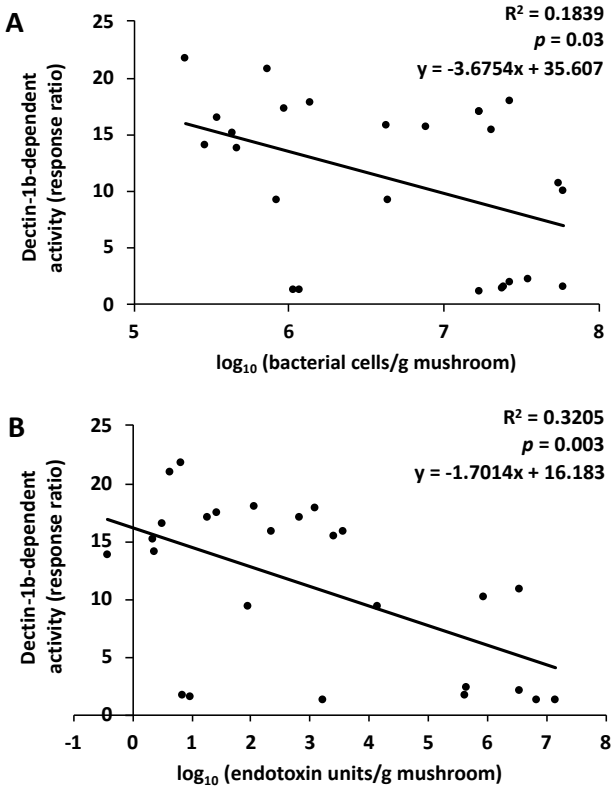
A
R² = 0.1839
p = 0.03
y = -3.6754x + 35.607
Dectin-1b-dependent activity (response ratio)
log10 (bacterial cells/g mushroom)
B
R² = 0.3205
p = 0.003
y = -1.7014x + 16.183
Dectin-1b-dependent activity (response ratio)
log10 (endotoxin units/g mushroom)

Figure 6

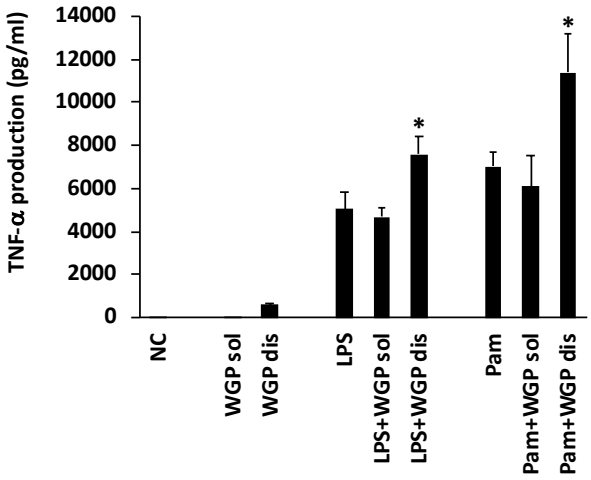
TNF-α production (pg/ml)
14000
12000
10000
8000
6000
4000
2000
0
*
*
NC
WGP sol
WGP dis
LPS
LPS+WGP sol
LPS+WGP dis
Pam
Pam+WGP sol
Pam+WGP dis

Figure 7

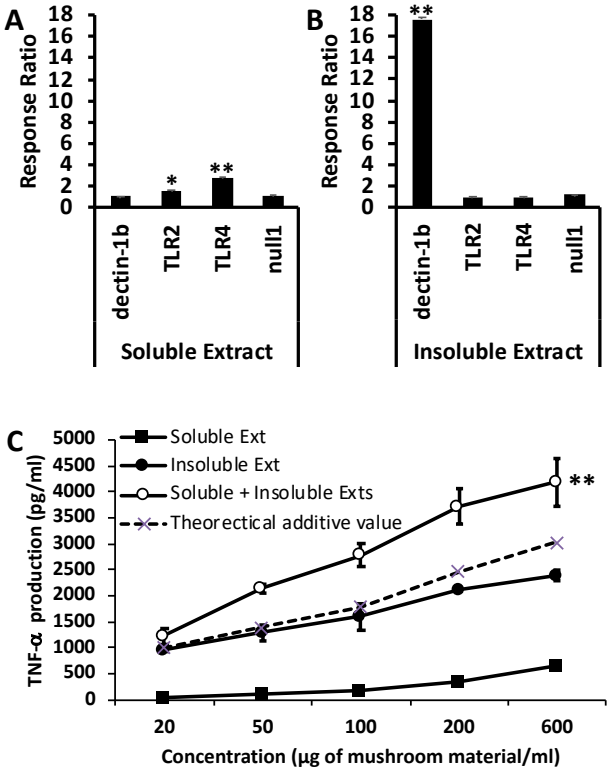
A
B
Response Ratio
18
16
14
12
10
8
6
4
2
0
*
**
dectin-1b
TLR2
TLR4
null1
Soluble Extract
Insoluble Extract
C
Soluble Ext
Insoluble Ext
Soluble + Insoluble Exts
Theorectical additive value
TNF-α production (pg/ml)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
20
50
100
200
600
Concentration (μg of mushroom material/ml)

Table of contents: graphic and sentence

Insoluble beta glucans and TLR agonists derived from colonizing microorganisms are responsible for *in vitro* macrophage activation by edible mushrooms.