Food & Function

PAPER

Cite this: *Food Funct.*, 2016, **7**, 4213

Bacterial components are the major contributors to the macrophage stimulating activity exhibited by extracts of common edible mushrooms†

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Received 20th April 2016,
Accepted 15th September 2016

DOI: 10.1039/c6fo00562d

www.rsc.org/foodfunction

Recent studies have indicated that a major contributor to the innate immune enhancing properties of some medicinal plants is derived from the cell wall components of bacteria colonizing these plants. The purpose of the current study was to assess if the bacteria present within edible and medicinal mushrooms substantially contribute to the innate immune stimulating potential of these mushrooms. Whole mushrooms from thirteen types of edible fungi and individual parts from *Agaricus bisporus* were analyzed for *in vitro* macrophage activation as well as bacterial lipopolysaccharides (LPS) content, cell load, and community composition. Substantial variation between samples was observed in macrophage activation (over 500-fold), total bacterial load (over 200-fold), and LPS content (over 10 million-fold). Both LPS content (ρ = 0.832, $p < 0.0001$) and total bacterial load (ρ = 0.701, $p < 0.0001$) correlated significantly with macrophage activation in the whole mushroom extracts. Extract activity was negated by treatment with NaOH, conditions that inactivate LPS and other bacterial components. Significant correlations between macrophage activation and total bacterial load (ρ = 0.723, p = 0.0001) and LPS content (ρ = 0.951, $p < 0.0001$) were also observed between different tissues of *Agaricus bisporus*. *Pseudomonas* and *Flavobacterium* were the most prevalent genera identified in the different tissue parts and these taxa were significantly correlated with *in vitro* macrophage activation (ρ = 0.697, $p < 0.0001$ and ρ = 0.659, p = 0.0001, respectively). These results indicate that components derived from mushroom associated bacteria contribute substantially to the innate immune enhancing activity exhibited by mushrooms and may result in similar therapeutic actions as reported for ingestion of bacterial preparations such as probiotics.

Introduction

A growing body of evidence supports the theory that bacterial communities within immune-enhancing botanicals are the principal source of components responsible for the activation of innate immune cells by extracts from these plants. Our prior research has demonstrated that bacterial lipopolysaccharides (LPS) and Braun-type lipoproteins were responsible for 97% of the *in vitro* macrophage activation exhibited by extracts of eight botanicals traditionally used to enhance immune function.[1] Variations in the levels of these two bacterial components are also responsible for the up to 200-fold difference in *in vitro* monocyte/macrophage activation potential exhibited by *Echinacea purpurea* and *Echinacea angustifolia* bulk commercial material.[2] We have found that *E. purpurea* samples contain bacterial loads ranging from 10^6 to 10^8 bacterial cells g^{-1} of dry material and that both bacterial loads and LPS content are strongly correlated with *in vitro* macrophage activity.[3] Other groups have reported similar findings. Endophytic bacteria contributed to the *in vitro* pro-inflammatory effects of *E. purpurea* crude extracts[4] and to the immunostimulatory activity of *Angelica sinensis*, one of the most potent herbs in the traditional East Asian formula Juzen-taiho-to.[5]

While plants are important as a source of immune-stimulating preparations, mushrooms also have a long history of medicinal use for enhancing immune function. This is sup-

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ported by studies that indicate that dietary ingestion of mushrooms or their extracts stimulates immune cells.[6] As with plants, mushrooms are colonized by communities of bacteria. However, most studies on mushroom-colonizing bacteria have focused on their presence from a food safety perspective[7] or on the bacteria that cause visible disease symptoms that can impact retail value.[8] Although reducing or eliminating potentially pathogenic bacteria associated with mushrooms is important, not all mushroom-colonizing bacteria represent a food safety issue. It is our hypothesis that the naturally occurring bacterial communities on the surface of and within mushrooms contribute substantially to their innate immune enhancing potential.

The aim of the current study was to assess the *in vitro* macrophage activation potential of extracts from an assortment of commercially obtained common edible mushrooms and to determine if differences in activity correlate with the bacterial load and LPS levels present in these products. In addition, bacterial community composition within each sample was analyzed using 16S rRNA gene pyrosequencing to determine if specific bacterial taxonomic groups correlated with *in vitro* macrophage activation exhibited by the mushroom extracts.

Materials and methods

Mushroom material

Thirteen types of edible mushroom samples (Table 1) were obtained from local supermarkets in Oxford, MS and Memphis, TN, USA. Mushroom samples were analyzed as whole material or dissected into different parts. For dissection, mushroom material (portobello and white button) were separated into four parts: stem tissue, gills, cap peel, and internal (represents mushroom cap with gills and cap peel removed). To mimic the culinary use of mushrooms, the 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). All samples were freeze-dried, ground into a fine powder and stored at −20 °C until analysis.

Extract preparation

Ground mushroom material (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 anti-inflammatory (inhibitors of macrophage activation). Ethanol extracted mushroom material was then dried overnight at 50–55 °C.

Crude extracts were prepared for assessment of macrophage enhancing activity by further extracting the dried ethanol extracted material with 0.5 mL of water containing 4% SDS at 98 °C for 1 hour. SDS was then removed using SDS-out reagent (Life Technologies, Grand Island, NY) in the presence of 1% octylglucoside. For assessment of LPS (endotoxin) content, crude extracts were prepared by further extracting the dried ethanol extracted material with 0.5 mL of water containing 2% SDS at 98 °C for 1 hour, followed by removal of SDS using SDS-out reagent.

Table 1 Total bacterial load, lipopolysaccharide (LPS) levels and *in vitro* macrophage activation potential exhibited by extracts of 13 different types of edible mushrooms

Sample #	Mushroom	Material	TNF-alpha[a] (pg ml^{-1})	LPS level[b]	Bacterial load (×10^6 cells per g)
1	Maitake (*Grifola frondosa*)	Fresh	19.6	7	0.22
2		Fresh	22.0	4	0.73
3	Bunashimeji (*Hypsizygus marmoreus*)	Fresh	18.3	3	0.34
4		Fresh	23.2	2	0.44
5	Bunapi (*Hypsizygus marmoreus*)	Fresh	24.5	2	0.29
6		Fresh	26.9	0.4	0.47
7	Oyster (*Pleurotus ostreatus*)	Fresh	70.0	1174	1.4
8		Fresh	85.5	112	27
9		Dried	50.8	225	4.2
10		Dried	333.9	3658	7.8
11	Crimini (*Agaricus bisporus*)	Fresh	134.4	9	24
12		Fresh	158.4	7	25
13	Shiitake (*Lentinula edodes*)	Fresh	150.0	19	17
14		Fresh	211.6	26	0.94
15	White button (*Agaricus bisporus*)	Fresh	1075.0	1626	17
16	Woodear (*Auricularia auricula-judae*)	Dried	1302.0	6 647 669	1.1
17		Dried	2165.6	14 265 954	1.2
18	Enoki (*Flammulina velutipes*)	Fresh	2112.8	668	17
19		Fresh	2610.2	2445	21
20	Morel (*Morchella esculenta*)	Dried	1864.4	89	4.4
21		Dried	5810.8	13 952	0.85
22	Lobster (*Hypomyces lactifluorum*)	Dried	4830.0	867 965	60
23		Dried	4881.9	3 412 805	55
24	Chanterelle (*Cantharellus cibarius*)	Fresh	4639.3	3 557 130	27
25		Fresh	6938.1	428 795	35
26	Portobello (*Agaricus bisporus*)	Fresh	9945.7	401 635	59

[a] *In vitro* macrophage activation potential measured by the amount of TNF-alpha secreted by RAW 264.7 cells exposed to extract from 40 μg of mushroom material per ml. The TNF-alpha value for untreated cells was 17.9 pg per ml. [b] LPS level expressed as endotoxin units per g of dried mushroom material.

Macrophage activation assay and limulus amebocyte lysate (LAL) assay

RAW 264.7 mouse macrophages (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% v/v FBS (Hyclone Laboratories, Inc., Logan UT) and amikacin (60 mg L^{-1}) at 37 °C, under 5% CO_2. Actively growing cells were suspended in culture medium at 0.5 million cells per mL and plated at a density of 1×10^5 cells per well in 96-well plates. Test samples were added to cells and after incubation for 18 h, the level of TNF-alpha in the cell culture supernatants was determined using ELISA (R&D Systems). Macrophage activation is reported as TNF-alpha production (pg ml^{-1}). Ultra pure *E. coli* LPS from *E. coli* 0111:B4 and Pam3CSK4 were purchased from InvivoGen (San Diego, CA). *S. cerevisiae* particulate beta-glucan (WGP® dispersible) was from Biothera (St. Paul, MN).

Endotoxin content of mushroom extracts was determined using the Pyrochrome® LAL assay with Glucashield® (1 → 3)-β-D-glucan Inhibiting Buffer (Associates of Cape Cod, Inc., East Falmouth, MA). Data is reported as endotoxin units (EU) per g of dried material and represents the average of determinations that were performed at least twice.

Determination of total bacterial load

Fifty milligrams of ground, freeze-dried mushroom material was rehydrated with 150 μL sterile water and DNA extracted using PowerPlant® Pro DNA isolation Kits (MoBio Laboratories, Carlsbad, CA), followed by a secondary clean-up using PowerClean® DNA cleanup kits (MoBio). The bacterial 16S rRNA gene from each sample was then amplified using primers 799f and 1492r as described previously.[3] Bacterial loads were estimated by comparing the intensity of the 735 bp 16S bacterial band from mushroom extracts to a standard curve of DNA extracted and amplified from known quantities of bacteria.[3]

Determination of bacterial community structure

Bacterial community structure was determined by bacterial tag-encoded FLX amplicon 454 pyrosequencing (bTEFAP)[9] conducted on the amplified 16S rRNA product from each sample, through a dedicated sequencing facility (MR DNA, Shallowater, TX). Bacterial primers 939f and 1392r[10,11] were used in the sequencing reaction, following conditions described previously.[12] Following PCR, products from different samples were mixed in equal concentrations and purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Danvers, MA). Samples were sequenced using Roche 454 FLX titanium instruments and reagents following the manufacturer's guidelines.

Raw pyrosequence data was transferred into FASTA files for each sample, along with sequencing quality files. Files were processed with the bioinformatics software Mothur[13] and analyzed following the procedures recommended by Schloss *et al.*[14] Briefly, sequences were denoised and trimmed to remove barcodes and primers. Chimeric sequences were removed and sequences were then aligned and classified according to those in the SILVA rRNA database,[15] after which any sequences classified as mitochondrial or chloroplast were removed from the dataset. The cell load of each bacterial taxon in mushroom samples were subsequently estimated from the proportion of sequencing reads of each taxon and the total bacterial load in each sample. Selected samples had their bacterial classification verified by subsampling 50–100 sequences from FASTA files and comparing them (BLAST) to sequences in the NCBI Genbank 16S rRNA database.

Statistical analysis

Statistical analyses were performed in JMP®11.2.0. Because data may not meet the requirements for parametric analyses, Spearman's Rank Correlation was performed to examine relationships between macrophage activation stimulatory activity exhibited by the mushroom extracts and both bacterial load (total and for each individual taxon) and LPS content. Differences in estimated total bacterial load between tissue parts for portobello and white button mushroom samples were determined using ANOVA followed by Tukey's Honestly Significant Difference test on $\log_{10}$ transformed data.

Results

The 13 types of edible mushrooms exhibited dramatic variation in the levels of *in vitro* macrophage activation, content of bacterial LPS and bacterial load. Extract activity (TNF-alpha production by RAW 264.7 cells) and LPS levels varied by over 500-fold and more than 10-million fold, respectively (Table 1). Bacterial load also varied between samples, with numbers ranging from 2.2×10^5 cells per g mushroom to 6.0×10^7 (Table 1). There was a significant relationship between total bacterial load and macrophage activation exhibited by the mushroom extracts (Fig. 1a, $\rho = 0.701$, $p < 0.0001$). Macrophage activation exhibited by extracts was also significantly related to levels of LPS (Fig. 1b; $\rho = 0.832$, $p < 0.0001$). Treatment of extracts with 0.5 M NaOH for 1 h at 98 °C reduced TNF-alpha production to the same levels as observed with NaOH treatment of Pam3CSK4 and ultra pure *E. coli* LPS (Fig. 2a). None of the NaOH treated extracts contained components that collaborated with Toll-like receptor 4 (TLR4) signaling to enhance TNF-alpha production in RAW 264.7 cells (Fig. 2b). The positive control, particulate beta-glucan from *S. cerevisiae*, is not inactivated by NaOH treatment and both untreated and NaOH treated beta glucan material collaborated with the TLR4 agonist ultra pure *E. coli* LPS to synergistically enhance TNF-alpha production (Fig. 2b).

Of the 26 mushroom samples analyzed, nine (from the bunapi, bunashimeji, maitake, and woodear varieties) did not yield sufficient bacterial DNA so that only 17 samples were analyzed by pyrosequencing. These samples yielded a total of 50 243 16S rRNA gene sequences ≥200 bp. None of these sequences were classified as mitochondrial or chloroplast. Following the removal of chimeric sequences (20.8% of total),

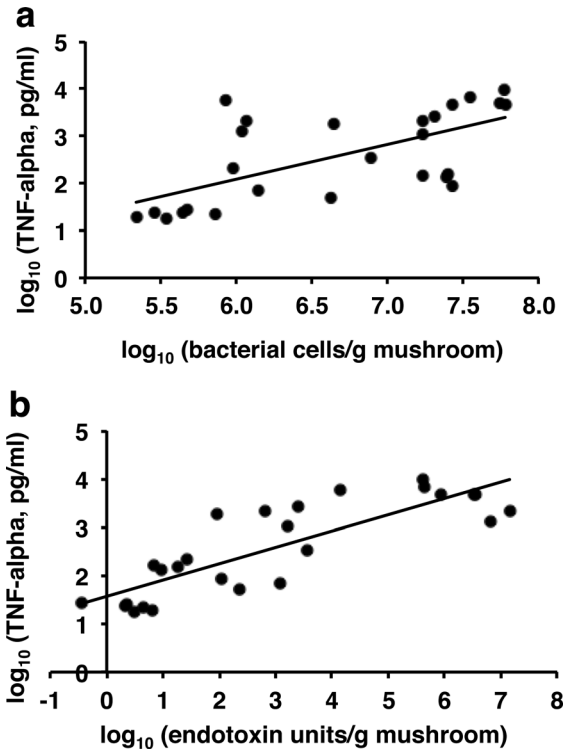

Fig. 1 Correlation of macrophage stimulatory activity exhibited by extracts from 13 different types of edible mushrooms with total bacterial load (a) and LPS content (b). Types of mushrooms and number of samples for each mushroom are detailed in Table 1.

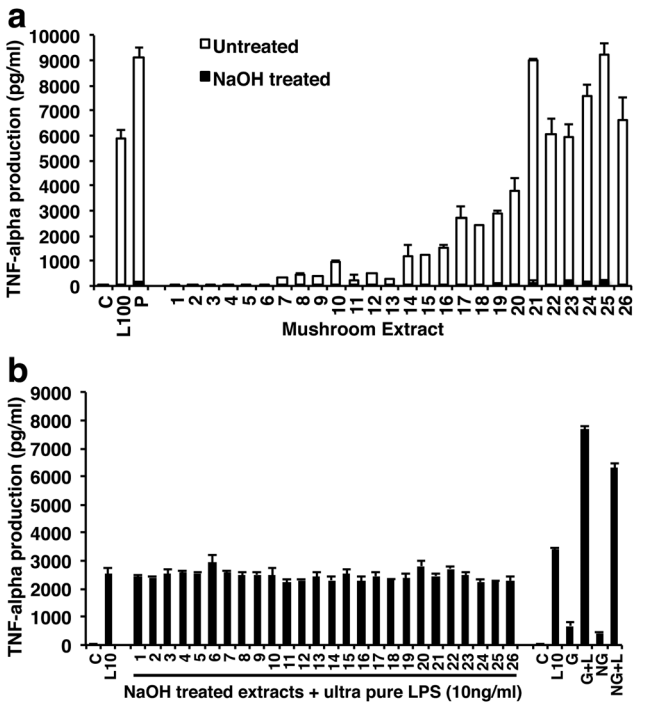

Fig. 2 *In vitro* macrophage stimulatory activity of extracts from 13 different types of edible mushrooms is due to components that are inactivated by treatment with NaOH. TNF-alpha production was evaluated in RAW 264.7 macrophages exposed to untreated and 0.5 M NaOH treated extracts (a), and NaOH treated extracts plus ultra pure LPS at 10 ng ml^{-1} (b). Pam3CSK4 (P) was tested at 100 ng ml^{-1} and ultra pure LPS at 10 ng ml^{-1} (L10) and 100 ng ml^{-1} (L100). *S. cerevisiae* particulate beta glucan (G) and NaOH treated beta glucan (NG) were evaluated at 100 μg ml^{-1} alone and together with ultra pure LPS at 10 ng ml^{-1} (G + L, NG + L). Numbers below bars correspond to sample numbers in Table 1. Concentrations of both untreated and NaOH treated samples in the culture medium represent extract from 100 (samples 1–15), 40 (samples 16–25) and 16 (sample 26) μg of mushroom material per ml. (C) refers to untreated cells.

39 810 partial 16S rRNA gene sequences (mean 2342 sequences per sample, but ranging from 767 to 7884) remained for subsequent analyses.

Representatives of at least seven distinct bacterial phyla were identified, as well as members of four of the major subphyla of Proteobacteria (Table 1S†). Proteobacteria were by far the most dominant phylum, accounting for over 80% of the recovered sequences. Of these, the majority of sequences were identified as belonging to the Gammaproteobacteria. Of the remaining phyla, only Bacteroidetes and Firmicutes accounted for >1% of the total number of sequences obtained, although some specific samples had greater proportions of a specific phylum. When the estimated cell load of individual phyla was related to macrophage activation, none of the phyla correlated significantly with TNF-alpha production ($p > 0.1$ for all).

At a finer taxonomic level, *Pseudomonas* (Gammaproteobacteria) was the only bacterial genus detected that composed at least 1% of the community in all 17 samples, and was the most abundant genera in 12 samples (Fig. 3 and Table 2S†). Sequences classified as *Pseudomonas* often dominated the community, and accounted for >90% of the bacterial community in four samples. Other abundant genera included *Flavobacterium* (Bacteroidetes), which was the most abundant genera detected in two samples (morel and one of the dried oyster mushroom samples), and unclassified members of the Enteric Bacteria cluster (Gammaproteobacteria), which was the most abundant genera detected in both enoki samples (Fig. 3 and Table 2S†). A total of 32 genera were identified that accounted for at least 1% of the sequences recovered from at least one sample (Table 2S†). Proportions of none of the abundant (>1% total) phyla or genera correlated significantly with macrophage activation (Table 2), although this was likely skewed by the lack of sequence data from nine samples, many of which showed low macrophage activation.

In addition to whole mushrooms, the variation in bacterial load in different tissue parts of *Agaricus bisporus* (white button and portobello mushrooms) was also evaluated. For portobello mushrooms the mean bacterial load in the different parts varied less than two-fold (Fig. 4; 2.8×10^7 to 4.8×10^7 cells per g) and no statistically significant difference in load was observed between the parts. In white button mushrooms, the mean bacterial load in stem, internal tissue and peel varied from 1.5×10^7 to 3.6×10^7 cells per g, whereas the bacterial load within the gills was almost two orders of magnitude lower

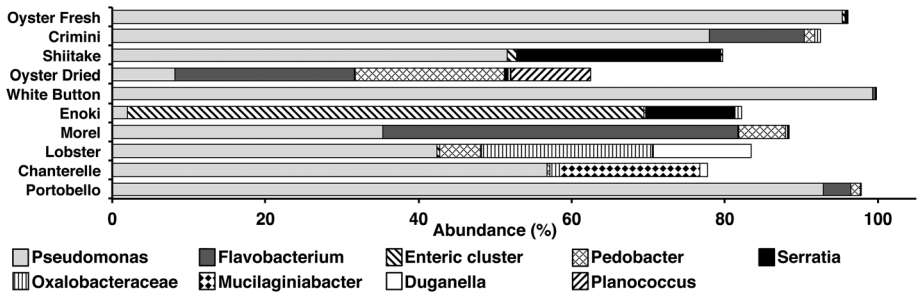

Fig. 3 Average bacterial community structure within various edible mushrooms. Mushroom material and number of samples for each mushroom is detailed in Table 1. Data represent most abundant taxa identified that accounted for over 10% of the sequences recovered from at least one sample.

Table 2 Statistics for Spearman's Rank Correlations between cell load of various bacterial groups and *in vitro* macrophage activation potential exhibited by mushroom samples. Details for the whole mushrooms and number of samples for each mushroom are summarize in Table 1

Bacterial group (total, phylum or genus)	Whole mushrooms			Tissue parts for *Agaricus bisporus*		
	ρ	p	n	ρ	p	n
Total bacteria	0.701	<0.0001	26	0.723	<0.0001	28
Proteobacteria	0.370	0.1437	17	0.694	<0.0001	28
Bacteroidetes	0.378	0.1353	17	0.652	0.0002	28
Pseudomonas	0.238	0.3582	17	0.697	<0.0001	28
Flavobacterium	−0.067	0.7983	17	0.659	0.0001	28

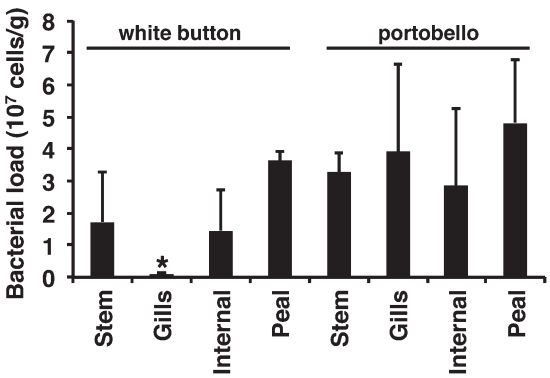

Fig. 4 Levels of bacterial load present within internal and external parts of white button (n = 3 for each part) and portobello mushrooms (n = 4 for each part). Internal part represents mushroom cap with external parts (gills and cap peel) removed. Stem tissue represents both internal and external tissue. Total bacterial load (cells per g dry material) was estimated using a PCR-based quantification method that we previously developed (Pugh *et al.*, 2013).[3] *Gills of white button had a significantly lower bacterial load than other white button tissues, p = 0.0104 (determined by ANOVA followed by Tukey's HSD test on $\log_{10}$ transformed data).

(8.8×10^5 g^{-1}), a difference that was statistically significant (p = 0.0104; Fig. 4).

As with the whole mushrooms, there was a significant correlation between total bacterial load and macrophage activation exhibited by extracts of the portobello and white button tissue parts (Fig. 5a, ρ = 0.723, p = 0.0001). LPS content within the tissue parts was also significantly correlated with *in vitro* macrophage activation potential (Fig. 5b, ρ = 0.951, p < 0.0001). Correlations between bacterial load and activation potential were slightly stronger when only external mushroom parts (gills and peel) were included in the analysis (ρ = 0.776, p = 0.0011), but there were no significant correlations between activity and bacterial load for internal tissue or stem tissue samples alone (ρ = 0.464 and ρ = 0.250, respectively).

Individual parts of both portobello and white button were dominated by the phylum Proteobacteria (especially Gammaproteobacteria), representing >96% of the sequences identified (Table 3S†). Of the remaining phyla that could be

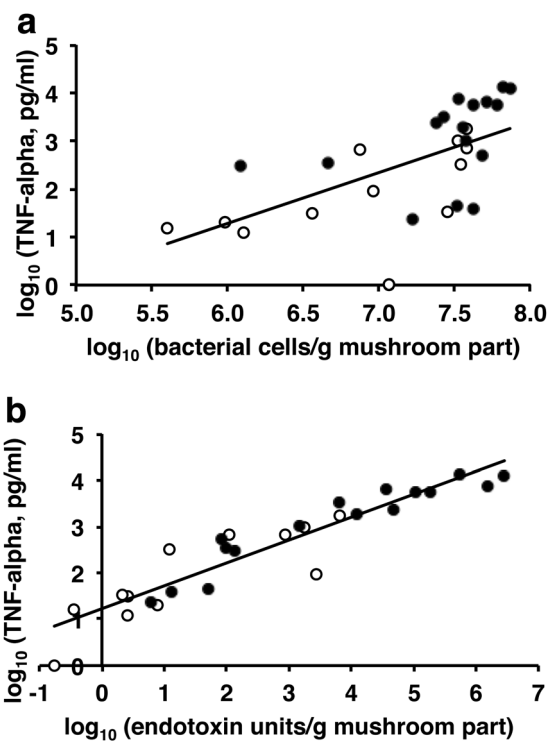

Fig. 5 Correlation of total bacterial load (a) and LPS content (b) with macrophage activation exhibited by extracts of white button (n = 12, indicated by white circles) and portobello parts (n = 16, indicated by black circles).

identified, only Bacteroidetes and Firmicutes, made up >1% of the bacterial sequences in portobello (2.87% Bacteroidetes) or white button (1.27% Firmicutes) samples. Overall, correlations with macrophage activation were higher within portobello and white button samples than when all 13 mushroom types were compared (Table 2). Correlations between macrophage activation and total bacterial load were similar in all 13 mushroom types and the *A. bisporus* samples specifically (Table 2). However, some bacterial taxa identified in *A. bisporus* parts correlated significantly with macrophage activation (Table 2).

At the phylum level, both Proteobacteria (ρ = 0.694, $p < 0.0001$) and Bacteroidetes (ρ = 0.652, p = 0.0002) correlated significantly with macrophage activation. *Pseudomonas* was the dominant genus in *A. bisporus*, ranging from 70–100% of the sequences identified in each sample (Table 3S†). The next most prevalent genus, *Flavobacterium*, made up 0–12% and 0–0.7% of the bacterial sequences in portobello and white button mushrooms, respectively (Table 3S†). As the most prevalent member of the Proteobacteria, *Pseudomonas* correlated highly with macrophage activation (ρ = 0.697, $p < 0.0001$) in *A. bisporus* samples, as did *Flavobacterium* (ρ = 0.659, p = 0.0001; Table 2).

Discussion

We evaluated 13 types of culinary mushrooms, seven of which (maitake, shiitake, oyster, bunashimeji, bunapi, woodear and enoki) have also been used traditionally for medicinal purposes. Maitake, shiitake and oyster mushrooms have been used to enhance immune function, and studies support this therapeutic effect.[16–18] Bunashimeji, bunapi and enoki mushrooms contain polysaccharides[19] or proteoglycans[20] that activate macrophages *in vitro*. Although *Agaricus bisporus* (white button, portobello, crimini) is generally used solely for culinary purposes, dietary supplementation of this mushroom enhances NK cell activity in mice.[21] We hypothesized that bacterial products derived from the bacteria that colonize these fungi are major contributors to the innate immune enhancing activity exhibited by these medicinal and culinary mushrooms. The strong correlations between *in vitro* macrophage activation (as determined by TNF-alpha production by RAW 264.7 macrophages) and LPS content and bacterial load of these samples support this hypothesis. These results are consistent with our previous research on immune enhancing plant botanicals showing that immune enhancing activity is strongly correlated with LPS content and bacterial load within *E. purpurea*.[3]

The substantial reduction (97–100%) in *in vitro* macrophage activation exhibited by the mushroom extracts after NaOH treatment indicates that the activity of these samples is due to bacterial components. The bacterial components LPS, Braun-type lipoproteins and lipoteichoic acid are inactivated by the base-dependent removal of ester- and amide-linked fatty acids. The low level of base-resistant activity exhibited by some of the NaOH-treated mushroom extracts could be due to beta glucans since these polysaccharides retain dectin-1 dependent activity following treatment with strong base (*e.g.* boiling in 10 M NaOH for 0.5 h).[22] NaOH treatment converts the triple-helix structure of high molecular weight (1,3)-beta-D-glucans to a partially opened triple-helix conformation[23] or single-helix conformation.[24,25] Since most research demonstrates that the partially opened triple-helix and single-helix conformations exhibit enhanced macrophage activation *in vitro*[23–25] compared to the triple-helix structure, the NaOH treatment of the mushroom extracts would not have decreased activity due to beta glucans. Although one study reported that TNF-alpha production from RAW 264.7 cells by *Grifola frondosa* (1,3)-beta-D-glucan was abolished by treatment with NaOH,[26] it is likely that the observed reduction in activity was due to inactivation of bacterial components derived from contamination of the test materials during the extraction/isolation procedure.

The combination of ultra pure LPS and a beta glucan result in a collaborative interaction that can be detected as an enhancement in TNF-alpha production by macrophages.[27] The lack of enhancement in TNF-alpha production observed with the NaOH treated mushroom samples evaluated in combination with ultra pure LPS may be due to the concentration of extract tested (appropriately 25 μg ml^{-1} and lower). By comparison, published studies typically assess activity of purified beta glucans at 100 μg ml^{-1}.[24,25] Even at 100 μg ml^{-1} of purified *S. cerevisiae* 1,3-beta glucan we only observed a moderate increase in TNF-alpha production (19% of levels achieved by ultra pure LPS tested at 10 ng ml^{-1}). Since a major portion of the activity detected in the mushroom extracts appears to be due to LPS and other bacterial substances, it will be important to determine whether contaminating bacterial components are partially or solely responsible for the activity of some of the mushroom derived polysaccharide preparations reported in the literature.

Production of TNF-alpha by RAW 264.7 cells was used to measure macrophage activation since this system detects the additive effect of bacterial LPS and Braun-type lipoproteins. Our previous research demonstrated that LPS and Braun-type lipoproteins are the most potent monocyte/macrophage activating components of bacteria and both contribute substantially to the *in vitro* monocyte/macrophage activation exhibited by extracts of *Echinacea.*[2] In animal models and human clinical trials we have demonstrated the therapeutic potential of one of these bacterial components using an extract enriched for Braun-type lipoproteins. In mice, oral ingestion of this extract exhibited a protective effect against influenza A (H1N1) viral infection[28] and enhanced *ex vivo* production of IgA and IL-6 from Peyer's patch cells as well as interferon-gamma production from spleen cells.[29] In two separate clinical trials dietary supplementation with this extract enhanced NK cell activity by 40 and 54%.[30] Similar therapeutic benefits on enhancing immune function are observed following oral ingestion of both live and heat killed probiotic bacteria.[31] The above studies suggest that consumption of bacterial components, whether derived from a bacterial preparation or from bacteria colonizing plants or mushrooms, can have therapeutically relevant effects on enhancing immune function.

Mushrooms used in the current study were purchased either fresh or dried from local supermarkets and therefore factors such as post-harvest events are unknown. It is conceivable that the high level of estimated total bacterial load in some of the mushroom samples could have originated from post-harvest contamination. However, any bacteria introduced post-harvest are likely to have been removed from the external surfaces of the fresh mushrooms as these samples were washed prior to use (the exception being oyster mushrooms, as label instructions indicated not to wash prior to consumption). For *Agaricus bisporus* the internal tissue contained high concentrations of bacteria that were comparable to, or higher than that observed within the external surfaces (peel and gills). These internally colonizing bacteria would have contributed substantially to the total bacterial load that we detected in the mushroom samples.

Gram-negative taxa were the most prevalent bacteria detected within mushroom samples, and these bacteria exhibited the strongest correlations with *in vitro* macrophage activation. Abundances of the phyla Proteobacteria and Bacteroidetes exhibited significant correlations with *in vitro* macrophage activation by white button and portobello mushrooms, whereas the next most abundant phyla detected, the Gram-positive Firmicutes and Actinobacteria, showed no significant relationships to *in vitro* macrophage activation. We have found more than 8000-fold variation in *in vitro* macrophage activation between different bacterial isolates obtained from plants,[32] so variation in the correlation between immune enhancing activity and the abundance of different bacterial taxa is not surprising. Plant-derived isolates identified as members of the Proteobacteria show the highest level of *in vitro* macrophage stimulatory activity, supporting the findings of the current study, and suggesting that members of this taxon may be important contributors to both botanical and mushroom immune stimulation.

Pseudomonas was the most commonly detected genus associated with mushrooms. A previous culture-based study also reported high numbers of *Pseudomonas* on mushroom samples.[7] Leff and Fierer[33] used 16S rRNA gene pyrosequencing to examine the bacterial communities on the surface of mushrooms, and also reported *Pseudomonas* sp. to be one of the most abundant bacterial genera present, accounting for 11% of the sequences obtained. Of the 20 most prevalent bacterial genera detected in the current study, only *Pseudomonas* and *Flavobacterium* displayed significant correlations to macrophage activity exhibited by portobello and white button mushroom. Although this indicates that *Pseudomonas* and *Flavobacterium* are important taxa that contribute to the *in vitro* macrophage activation potential of the mushroom extracts, none of the bacterial taxa showed significant correlations when all the sequenced mushroom types were analyzed together. However, the lack of sufficient quantities of DNA for sequencing from nine of the mushroom samples resulted in a lack of community composition data from several low activity samples, which may have limited the correlation results. The lack of significance also suggests that other taxa are also important contributors to the activity exhibited by the mushroom extracts. There may also be significant differences in activity between bacterial species of the same genus; a potential variable that could not be evaluated since the bioinformatics pipeline and short read length of pyrosequencing only allowed absolute identification of taxa to the genus level. BLAST searches of some sequences suggested that those identified as *Pseudomonas* or *Flavobacterium* could actually represent six or more species of each genus, with the more common species varying between samples, supporting the idea that there could be species level variation across different samples. Different macrophage activity between different species or even strains of a bacterial genus, might therefore explain some of the variation between samples.

Although *Pseudomonas* was present in all samples analyzed (and was the most predominant taxa in most samples), there was also variation in the bacterial community composition between the different mushrooms. One factor that may contribute to the differences in community structure between mushrooms is that some fungi contain antimicrobial substances. Extracts of Shiitake mushrooms have been reported to possess antimicrobial activity against species of *Pseudomonas*[34] and this may explain why the bacterial community in the Shiitake samples examined in this study was composed of only 43–59% *Pseudomonas*, compared to more than 90% of the community in some of the other mushroom types that were analyzed. Oyster and morel mushroom varieties have also been found to display antimicrobial activity against several bacteria, including *Pseudomonas*.[35] Sequences identified as *Pseudomonas* accounted for <50% of those derived from morel samples and <11% of two out of three sequenced oyster samples. The presence and type of naturally occurring antimicrobial agents is likely to differ between mushroom varieties, and this could influence the composition of the bacterial community in different mushroom types and the level of bacterial components contributing to extract activity.

Conclusion

The presence of bacteria in mushrooms is usually investigated from the standpoint of freshness and food safety. However, the current study examined the entire bacterial community composition within mushrooms, not just potential pathogens or organisms involved in spoilage. As with our previous research on plant botanicals,[1–3] the results reported here indicate that components derived from mushroom associated bacteria contribute substantially to the innate immune enhancing activity exhibited by extracts of common medicinal and culinary mushrooms. This link between bacterial components in edible mushrooms and enhanced immune function provide new insight into the significance of bacterial colonization of food products.

Abbreviations

LPS	Lipopolysaccharide
rRNA	Ribosomal ribonucleic acid
LAL	Limulus amebocyte lysate
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin units
DNA	Deoxyribonucleic acid
ANOVA	Analysis of variance.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

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 a grant from the USDA, Agricultural Research Service Specific Cooperative Agreement No. 58-6408-1-603.

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