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## Original Research

# Consuming *Lentinula edodes* (Shiitake) Mushrooms Daily Improves Human Immunity: A Randomized Dietary Intervention in Healthy Young Adults

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**Key words:** mushroom, immunity,  $\gamma\delta$ -T cell, NK-T cell, cytokine

**Background:** Mushrooms are widely cited for their medicinal qualities, yet very few human intervention studies have been done using contemporary guidelines.

**Objective:** The aim of this study was to determine whether consumption of whole, dried *Lentinula edodes* (shiitake) mushrooms could improve human immune function. Primary objectives were to ascertain whether *L. edodes* consumption would improve  $\gamma\delta$ -T cell proliferation and activation responses, quantify a dose response, and elicit cytokine secretion patterns. Secondary objectives included determining changes in natural killer T (NK-T) cell proliferation and activation, secretory immunoglobulin A (sIgA) in saliva, and C-reactive protein (CRP) in serum.

**Design:** Fifty-two healthy males and females, aged 21–41 years, participated in a 4-week parallel group study, consuming either 5 or 10 g of mushrooms daily. Each subject had blood drawn before and after 4 weeks of daily *L. edodes* consumption. Saliva and serum were also collected. Peripheral blood mononuclear cells were cultured in autologous serum for 24 hours or 6 days, stained, and examined by flow cytometry.

**Results:** Eating *L. edodes* for 4 weeks resulted in increased *ex vivo* proliferation of  $\gamma\delta$ -T (60% more,  $p < 0.0001$ ) and NK-T (2-fold more,  $p < 0.0001$ ) cells. Both cell types also demonstrated a greater ability to express activation receptors, suggesting that consuming mushrooms improved cell effector function. The increase in sIgA implied improved gut immunity. The reduction in CRP suggested lower inflammation. The pattern of cytokines secreted before and after mushroom consumption was significantly different; consumption resulted in increased interleukin (IL)-4, IL-10, tumor necrosis factor (TNF)- $\alpha$ , and IL-1 $\alpha$  levels, a decreased macrophage inflammatory protein-1 $\alpha$ /chemokine C-C ligand 3 (MIP-1 $\alpha$ /CCL3) level, and no change to IL-6, IL-1 $\beta$ , MIP-1 $\beta$ , IL-17 and interferon (IFN)- $\gamma$  levels.

**Conclusions:** Regular *L. edodes* consumption resulted in improved immunity, as seen by improved cell proliferation and activation and increased sIgA production. The changes observed in cytokine and serum CRP levels suggest that these improvements occurred under conditions that were less inflammatory than those that existed before consumption.

## INTRODUCTION

There are thousands of species of mushrooms, although only about 20 types of mushrooms are cultivated commercially for culinary purposes. The mushroom *Lentinula edodes*, commonly known as shiitake, is cultivated for both its culinary and medicinal qualities. *In vitro* and animal studies have indicated

the potential for immune-modulating activity; however, very few human studies have been performed.

The research in this lab has focused on the effect of diet on the function of the  $\gamma\delta$ -T cell. This cell has a T cell receptor (TCR) of gamma and delta chains that are similar to the alpha and beta chains of the more common circulating T cells. However, unlike  $\alpha\beta$ -T cells that recognize protein antigens,  $\gamma\delta$ -T

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This trial was registered at <http://www.clinicaltrials.gov> as NCT01398176.

Abbreviations: sIgA = secretory IgA, CRP = C-reactive protein, PBMC = peripheral blood mononuclear cells, NK-T = natural killer T cell, MFI = mean fluorescence intensity, MIP-1 $\alpha$  = macrophage inflammatory protein-1 $\alpha$ , CCL3 = chemokine C-C ligand 3.

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cells recognize small phospho-antigens that do not require the major histocompatibility complex for processing [1,2]. The majority of  $\gamma\delta$ -T cells are located in the epithelial lining of the gut, lungs, and genitourinary tract, where they act as a first line of immunological defense [3,4]. Although they are T cell lymphocytes, they behave more like a cell of the innate immune system.

The natural killer T (NK-T) cell is also considered an innate lymphocyte. Unlike the NK cell, the NK-T cells develop in the thymus and express CD3 on their surface. High NK-T concentrations are found in the liver. NK-T cells do not require sensitization to spontaneously lyse tumor cells. Currently, little is known about the interaction of diet with these cells.

The immunomodulating effect of shiitake mushrooms has been investigated in many *in vitro* [5–7] and *in vivo* models [8–11]. *Agaricus bisporus* (white button) mushrooms have also been shown to influence immunity in mice [12–14].

Reports of immunomodulation by any mushroom in humans are scarce, and those using *L. edodes*, even fewer. In a pilot study, Yamaguchi et al. [15] gave *L. edodes* mycelia extract to patients undergoing chemotherapy; an increase in NK cell activity and a decrease in immunosuppressive acidic protein levels were observed. One double-blind, crossover, placebo-controlled intervention study was performed with 42 healthy, elderly individuals. In that study, the soluble  $\beta$ -glucan extracted from *L. edodes* (lentinan,  $\beta$ -1,3;1,6-glucan) was consumed for 6 weeks and resulted in an increase in the number of circulating B cells but no change in immunoglobulins, complement proteins, or serum levels of cytokines [16].

The aim of this study was to determine whether the consumption of dried *L. edodes* mushrooms could improve human immune function, particularly regarding innate lymphocytes. Primary objectives of the study were to determine whether *L. edodes* consumption would improve  $\gamma\delta$ -T cell proliferation and activation responses, to define any dose response to the amount of mushrooms consumed, and to obtain data on cytokine networks. Additional objectives included determining changes to NK-T cell proliferation and activation, secretory immunoglobulin (sIgA), and serum C-reactive protein (CRP) levels, and memory (CD45RO<sup>+</sup>) and naïve (CD45RA<sup>+</sup>)  $\gamma\delta$ -T cell population numbers after mushroom consumption.

## SUBJECTS AND METHODS

### Subjects

After receiving approval from the University of Florida Internal Review Board, 66 generally healthy males and females, 21–41 years of age, were recruited and screened, by phone or in person, to participate in a 4-week, parallel-group study. To be eligible for enrollment, subjects could not be

vegan or vegetarian and had to agree to have blood drawn and saliva collected twice; stop consumption of tea, flavonoid-containing supplements, antioxidant supplements, or probiotics in advance; and, during the study, not consume more than 14 glasses of alcoholic beverages per week or more than 7 servings of fruits and vegetables per day. In addition, eligible subjects could not be on immunosuppressive drugs or antibiotics, have hypertension that required medication, chronically use nonsteroidal anti-inflammatory drugs, or have an ongoing infection. Women who were pregnant, lactating, or on hormone therapy did not meet the study inclusion criteria. Subjects were asked to keep a log of their illnesses during the study. Written consent was obtained from all enrolled subjects.

### Study Design

The study was conducted during the months of September and October of 2011. Eligible subjects reported to the clinical laboratory in the Food Science and Human Nutrition Building on the University of Florida campus, where consent was obtained and they were enrolled in the study by trained personnel. Once enrolled, participants were randomly assigned, using a computerized random number generator, to consume either 5 g ( $n = 26$ ) or 10 g ( $n = 25$ ) of dried *L. edodes* mushrooms every day for 4 weeks. These dried amounts were equivalent to one (3-oz.) or 2 (6-oz.) servings of fresh mushrooms based on 5 medium mushrooms as one serving [17] and a 90%–95% water content.

A 4-week supply of daily packets of dried mushrooms, either whole or ground, was given to subjects along with instructions for preparation, which included sufficient rehydration and cooking. Subjects received half of their mushrooms as ground and half as whole. The mushrooms were obtained with the help of a reputable local mushroom grower, who identified and sourced the dried shiitake mushrooms for the study.

There were 2 blood draws during the study: one before (baseline) and one after 4 weeks of mushroom consumption. Blood was drawn from fasting subjects by a trained phlebotomist and used for peripheral blood mononuclear cells (PBMC) isolation and to obtain serum.

In addition, subjects were instructed to complete daily illness and consumption logs over the course of the study. Illness logs were used to determine whether the groups differed in the pattern of illnesses, not as a health outcome. To help ensure compliance ( $\geq 80\%$  consumption), the study coordinator corresponded weekly with participants through e-mail. Subjects returned to the clinical lab at the end of the 4 weeks for the final blood draw, turned in their illness logs, and completed a questionnaire that asked about any side effects experienced during the intervention, supplement use (vitamins and minerals), and self-reported compliance. Overall compliance was determined by the quantity of mushrooms returned, as well as the self-reported data.

### **PBMC Isolation and Autologous Serum**

At each blood draw, 8 mL of blood was collected into a vacuum tube containing L-heparin; 3 mL was drawn into a serum activator tube (Vacutainer, BD, Franklin Lakes, NJ) and processed within 2 hours.

Serum tubes were kept chilled and then centrifuged (2000 × g, 10 minutes, 4°C). Serum was separated for use in the PBMC cultures, and additional aliquots were stored at –80°C for CRP analysis.

Whole blood was diluted 1:1 with 0.9% NaCl, layered over Lympholyte H density gradient separation medium (Cedarlane Laboratories Ltd., Burlington, NC), and centrifuged (800 × g, 20 minutes, 20°C). The PBMC band was removed and washed twice (1250 × g, 10 minutes, 4°C) in RPMI-1640 medium (Mediatech, Inc., Manassas, VA). The cell pellets were resuspended in clear RPMI 1640 medium containing 100,000 U/L penicillin, 100 mg/L streptomycin, 0.25 mg/L fungizone, 50 mg/L gentamicin, 2 mmol/L L-glutamine, and 25 mmol/L HEPES buffer, and cell counts were acquired using an automated cell counter (Nexcelom Bioscience LLC, Lawrence, MA).

### **Cell Culture**

PBMC ( $1 \times 10^6$ /well) were cultured in one well of each of two 24-well plates, in clear RPMI 1640 medium with 10% autologous serum and PHA-L (10 µg/mL), interleukin (IL)-2 (1 ng/mL), and IL-15 (1 ng/mL). The plates were incubated for 24 hours or 6 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After 24 hours, the cell culture media was harvested from the wells of one plate and frozen at –80°C for cytokine analysis. Cells were collected and stained for flow cytometry at 24 hours and 6 days.

### **Flow Cytometry**

Before and after mushroom consumption, PBMC were analyzed by flow cytometry at 3 time points: day 0 (uncultured) and 24 hours and 6 days of culture. Fluorescent antibodies to cell surface markers were obtained from Biolegend and eBioscience (San Diego, CA). Cells and antibodies were incubated together at 4°C in the dark for 30 minutes and the cells were washed and fixed with 2% formaldehyde. Cells were read within 48 hours on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software version 10.0.6 (Tree Star Inc., Ashland, OR).

On day 0, freshly isolated PBMC ( $5 \times 10^5$ ) in flow-staining buffer (phosphate-buffered saline with 2% fetal bovine serum) were stained with fluorescent antibody panels to determine baseline population and activation levels for γδ-T (CD3-PE, γδTCR-FITC, CD69-AF647) and NK-T (CD3-PE, CD56-AF647, CD314-FITC) cells. After 24 hours of culture, PBMC were stained using the same γδ-T and NK-T cell antibody

panels to determine the levels of activation for each cell type. After 6 days of culture, PBMC were stained to assess γδ-T (CD3-PE, γδTCR-FITC) and NK-T (CD3-PE, CD56-AF647) cell proliferation and for levels of γδ-T naïve and memory cells (γδTCR-FITC, CD45RA-AF700, CD45RO-PE). Proliferation and phenotype prevalence results are expressed as a population percentage, and activation results are expressed as mean fluorescence intensity. The shift in fluorescence intensity of the population of cells is interpreted as an increase or decrease in the number of receptors expressed on the cell surface.

### **Cytokine Determination**

The 24-hour cell culture fluids were assayed to determine the levels of 10 cytokines (IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-17, interferon [IFN]-γ, macrophage inflammatory protein-1α/chemokine C-C ligand 3 [MIP-1α/CCL3], MIP-1β, and tumor necrosis factor [TNF]-α), using a Human Cytokine Multiplex Immunoassay kit, according to manufacturer's directions (Millipore Corp., Billerica, MA). The beads were analyzed on a Luminex 200 instrument (Luminex Corp., Austin, TX) with xPONENT 3.1 software. Data are expressed in milligrams per liter.

### **Saliva Collection and sIgA Determination**

At each of the 2 blood draws, subjects were instructed to rinse their mouth thoroughly with water for 20 seconds. Following the rinse, subjects waited for 10 minutes and then used a half straw to transfer their saliva into a 15-mL conical tube during a 2-minute period. The saliva was kept cold and clarified by centrifugation (2300 × g, 10 minutes, 4°C), and aliquots were stored at –80°C until assayed. The amount of sIgA in salivary samples was measured by ELISA, using a commercially available kit (ALPCO Immunoassays, Salem, NH) following manufacturer's instructions. Determined levels of sIgA are expressed as both micrograms per milliliter and micrograms per minute.

### **Serum CRP**

Frozen serum samples were thawed and analyzed for total CRP using a Human C-Reactive Protein Quantikine ELISA kit (R&D Systems, Minneapolis, MN), as directed by the manufacturer. Total CRP concentrations are expressed in nanograms per milliliter.

### **Power Analysis**

Based on the proliferative capacity of the γδ-T cells in culture from previous studies [7,9–11], a sample size of 15 individuals per group would be needed to achieve 80% power at a 5% significance level to detect a 15% increase in the percentage of γδ-T+, CD3+ cell population after culturing. To account for dropouts, 25 subjects were recruited for each group.

## Statistics

For each subject, the outcomes were measured at baseline and postintervention. In addition, for those outcomes for which activation or proliferation was of interest, at each time period (baseline and postintervention) the initial and 24-hour (6-day) measurements were also collected. Hence, overall, for some outcomes we had several repeated observations on each subject. For those outcomes that had multiple measurements over time within each time period, the model was a repeated measures linear model with a non-zero compound symmetry covariance for observations on the same subject and with fixed effects of treatment, time period, and, where appropriate, culture time (uncultured and 24 hours or 6 days). To adjust for variables likely to have an effect on the outcomes but that were not controlled for during randomization, we included covariates for gender and the continuous variables body mass index (BMI) and age. The analyses proceeded in a hierarchical manner starting with the full model with all variables and all interactions of each variable with treatment, time period, and culture time included. The residuals were checked and, if needed, a log-transformation of the response was performed and the full model was rerun on the transformed data.

Cytokines, sIgA, CRP, and memory/naïve outcomes were analyzed using a general linear mixed model that included time period (baseline or postintervention) and treatment as fixed effects and random effect of subject to capture the repeated measurements on the same subject. Additional covariates included age, gender, and BMI and all 2-way interactions of these with treatment and time.

For both modeling approaches, nonsignificant terms were dropped from the model one by one starting with interactions and those with the largest *p* values. The model was rerun each time a variable or interaction was removed and checked again for further removals. The final model included only those covariates (age, BMI, and gender) that were statistically significant, plus time, culture time, and treatment. Each analysis was done on the intent to treat population and then repeated on the compliant group. The compliant models never differed from intent to treat models; therefore, only the intent to treat model is reported.

Means and SEs were reported for those fixed effects that were found to be significant and if the outcome required log-transformation, the means were back-transformed and the SEs for the back-transformed means were estimated using the Delta method [18].

## RESULTS

### Participants

Sixty-six individuals were assessed for eligibility, but 14 were excluded because they either declined participation or did not meet inclusion/exclusion criteria (Fig. 1). Fifty-two individuals were randomized to consume either one or 2

servings of mushrooms using a random number generator. One participant randomized to the 2-serving group was removed from the study due to participation in a conflicting study. Two individuals from the one-serving group dropped out due to a dermatitis associated with undercooked *L. edodes* mushrooms, and one individual was unable to return for the second blood draw. Three individuals in the 2-serving group dropped out due to nausea and gastrointestinal distress, and 3 subjects could not return for the second blood draw. This resulted in *n* = 23 in the one-serving group and *n* = 19 in the 2-serving group for analysis. Lack of compliance was determined for 2 individuals in the one-serving group and one individual in the 2-serving group. Intent to treat analysis versus per protocol analysis found no statistical difference; therefore, analysis on the intent to treat population has been reported. There was only one measurement that was statistically different between serving sizes (IL-4); therefore, the results of the 2 serving sizes are pooled and the difference between before and after mushroom consumption was the primary index of statistical difference. The demographics of the population that were allocated to intervention are shown in Table 1. There were no differences seen in the incidence and duration of self-reported illnesses during the study between individuals consuming either one or 2 servings of mushrooms (Table 2).

### Cell Proliferation and Activation

Stimulated  $\gamma\delta$ -T cells were able to proliferate to a greater extent *ex vivo*, after *L. edodes* were consumed for 4 weeks (Table 3). Statistically, the main effect of time (baseline vs 4 weeks) was significantly greater at 4 weeks. Post hoc analysis showed a significant interaction between time and culture time (uncultured, 24 hours, 6 days). Before mushroom consumption, stimulated cells proliferated within 24 hours to levels that were significantly greater than the uncultured cells, but the increase was not sustained during the 6 days of culture. After *L. edodes* were eaten, however,  $\gamma\delta$ -T cells maintained proliferation levels over the 6-day period.

The expression of CD69 on  $\gamma\delta$ -T cells is an indication of their potential for activation. Expression of CD69 in uncultured cells was not statistically different at the 2 time points (Table 3), whereas stimulation for 24 hours with a mitogen resulted in an increase both before and after consumption of *L. edodes*. However, after 4 weeks of mushroom consumption, the increase seen was significantly greater than that of baseline cells.

NK-T cell proliferation was also significantly greater at 4 weeks (Table 3). Post hoc analysis (Time  $\times$  Culture time) indicated a significant reduction in the percentage of this type of cell in the CD3+ population in uncultured cells. However, following mushroom consumption, a significant percentage increase was seen over baseline values after 24 hours and 6 days of culture and stimulation.

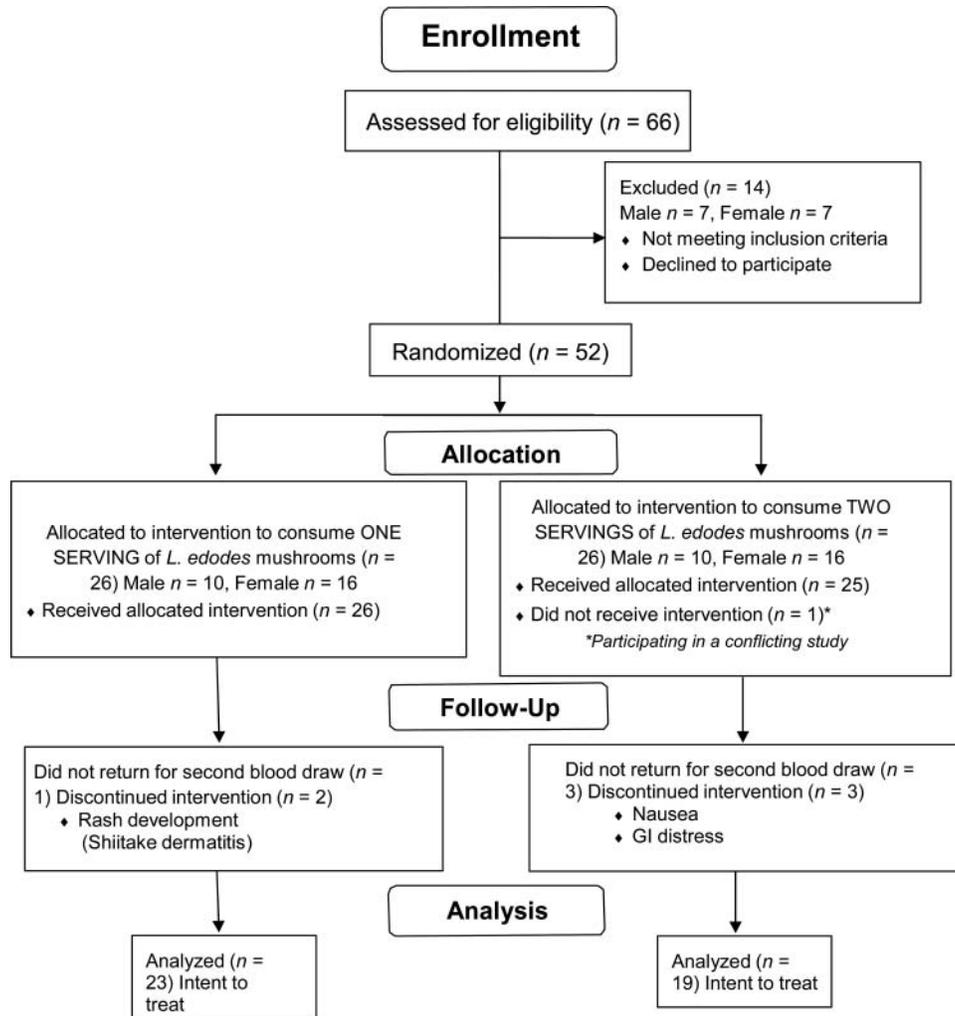


Fig. 1. Flowchart of subject eligibility, randomization, follow-up, and analysis.

Surface indicators of NK-T cell activation increased significantly from baseline to 4 weeks. Post hoc analysis of this data (Time × Culture time) showed significant differences in the levels of the activation marker. When baseline levels of marker expression on uncultured cells were compared with uncultured cells at 4 weeks, there was a substantial

reduction in mean fluorescence intensity, whereas the comparison of cells stimulated and cultured for 24 hours indicated a greater than 10-fold increase in marker expression between baseline and 4-week values.

Stimulating PBMC with a mitogen in culture for 6 days after subjects had consumed mushrooms for 4 weeks resulted

Table 1. Population Demographics of Subjects Allocated to Consume *L. edodes* Mushrooms<sup>1</sup>

	One Serving <sup>2</sup>		Two Servings	
	Female (n = 16)	Male (n = 10)	Female (n = 16)	Male (n = 9)
Age	22.8 ± 2.1 <sup>3</sup>	24.8 ± 6.4	25.5 ± 6.3	24.4 ± 2.9
Weight (kg)	57.3 ± 8.9	74.0 ± 6.7	60.1 ± 6.6	74.7 ± 7.1
Height (m)	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1
BMI (kg/m <sup>2</sup> )	21.7 ± 2.3	25.1 ± 2.2	22.4 ± 2.2	23.3 ± 2.4

BMI = body mass index.

<sup>1</sup>Dried mushrooms were prepared and consumed daily for 4 weeks.

<sup>2</sup>Mushroom serving size: One serving is defined as 3 oz. or 85 g fresh weight, equivalent to 5 g dried; 2 servings = 10 g dried [17].

<sup>3</sup>Mean ± SEM. Determined by 2-way analysis of variance.

**Table 2.** Self-Reported Illness in Subjects Consuming *L. edodes* Mushrooms<sup>1</sup>

Group	Incidence <sup>2</sup>	Duration <sup>3</sup>
One serving	18	38
Two servings	18	40
	$p = 0.739^4$	$p = 0.988$
	$z = -0.333$	$z = -0.0146$

<sup>1</sup>Subjects consumed either one (5 g) or 2 (10 g) servings of dried, prepared mushrooms daily for 4 weeks.

<sup>2</sup>Incidence is the number of times the subject reported at least one symptom associated with a cold, the flu, or an allergy.

<sup>3</sup>Duration is the total number of days with at least one symptom reported.

<sup>4</sup> $p$  determined by  $z$ -test of proportions; Yates correction applied.

in both significantly more naïve  $\gamma\delta$ -T cells and fewer memory  $\gamma\delta$ -T cells as a percentage of the entire CD3+ cell population when compared to baseline levels (Table 4).

### Biomarkers of Immunity and Inflammation

The amount of sIgA in the saliva and CRP in the serum are shown in Table 5. Levels of sIgA were significantly greater in the saliva after mushrooms were eaten for 4 weeks than at baseline. The average concentration of serum CRP for all participants was within normal range at baseline. After eating mushrooms for 4 weeks, CRP levels detected in the serum were reduced significantly by around 30%.

### Cytokine Production

Table 6 shows the concentrations of cytokines secreted into PBMC culture medium during 24 hours of mitogen stimulation. The quantities of IL-4, IL-10, IL-1 $\alpha$ , and TNF- $\alpha$  secreted were all significantly greater after mushroom consumption, whereas MIP-1 $\alpha$ /CCL3 secretion was significantly lower. The concentrations of IL-1 $\beta$ , IL-17, IFN- $\gamma$ , and MIP-1 $\beta$  in 24-hour culture fluids at 4 weeks were no different from those seen at baseline.

**Table 3.** Proliferation and Activation of Stimulated<sup>1</sup> Innate Lymphocytes before and after *L. edodes* Consumption<sup>2</sup>

Type		Time	Time $\times$ Culture Interactions			
			Uncultured	24 Hours	6 Days	
$\gamma\delta$ -T	Proliferation % of CD3+	Baseline	15.5 $\pm$ 0.6 <sup>3</sup>	12.4 $\pm$ 0.7 <sup>B</sup>	19.3 $\pm$ 1.5 <sup>A</sup>	14.9 $\pm$ 0.8 <sup>B</sup>
		4 weeks	19.2 $\pm$ 0.6	12.7 $\pm$ 0.8 <sup>B</sup>	22.5 $\pm$ 1.5 <sup>A</sup>	22.4 $\pm$ 0.8 <sup>A</sup>
		$p$ value	T: <0.0001	T $\times$ C: 0.8150	T $\times$ C: 0.1368	T $\times$ C: <0.0001
	Activation <sup>4</sup> mean fluorescence intensity	Baseline	4899 $\pm$ 456	755 $\pm$ 61 <sup>C</sup>	9044 $\pm$ 851 <sup>B</sup>	
		4 weeks	7411 $\pm$ 639	998 $\pm$ 85 <sup>C</sup>	13,824 $\pm$ 1194 <sup>A</sup>	
		$p$ value	T: <0.0001	T $\times$ C: 0.1644	T $\times$ C: <0.0001	
NK-T	Proliferation % of CD3+	Baseline	10.5 $\pm$ 0.9	12.6 $\pm$ 0.9 <sup>C</sup>	12.8 $\pm$ 0.9 <sup>C</sup>	5.9 $\pm$ 0.9 <sup>D</sup>
		4 weeks	14.4 $\pm$ 0.9	5.5 $\pm$ 0.9 <sup>D</sup>	21.2 $\pm$ 0.9 <sup>A</sup>	16.6 $\pm$ 0.9 <sup>B</sup>
		$p$ value	T: <0.0001	T $\times$ C: <0.0001	T $\times$ C: <0.0001	T $\times$ C: <0.0001
	Activation <sup>5</sup> mean fluorescence intensity	Baseline	264 $\pm$ 37	314 $\pm$ 49 <sup>C</sup>	215 $\pm$ 34 <sup>C,D</sup>	
		4 weeks	1236 $\pm$ 50	197 $\pm$ 31 <sup>D</sup>	2275 $\pm$ 183 <sup>A</sup>	
		$p$ value	T: <0.0001	T $\times$ C: <0.0001	T $\times$ C: <0.0001	

NK-T = natural killer T cells, T = time, T $\times$ C = post hoc interaction.

<sup>1</sup>PBMC were cultured in RPMI-1640 with 10% autologous serum, PHA-L (10  $\mu$ g/mL), IL-2 (1 ng/mL), and IL-15 (1 ng/mL).

<sup>2</sup>Subjects consumed either one (5 g) or 2 (10 g) servings of dried, prepared mushrooms daily for 4 weeks. Data from both serving groups were combined for analysis.

<sup>3</sup>Mean  $\pm$  SEM. A repeated measures linear model with a non-zero compound symmetry covariance for observations on the same subject and with fixed effects of treatment, time period. Values with different letters are significantly different.

<sup>4</sup>Mean fluorescent intensity of CD69 on the  $\gamma\delta$ TCR+, CD3+ cell population.

<sup>5</sup>Mean fluorescent intensity of NKG2D (CD314) on the CD56+, CD3+ cell population.

**Table 4.** Prevalence of Memory and Naïve Phenotypes in Cultured PBMC<sup>1</sup> before and after *L. edodes* Consumption<sup>2</sup>

Phenotype	Cell Surface Markers	Baseline	4 Weeks	$p$ Value
Memory	$\gamma\delta$ TCR-FITC/CD45RO-PE	45.7 $\pm$ 1.5 <sup>3</sup>	31.0 $\pm$ 1.5	T: <0.0001
Naïve	$\gamma\delta$ TCR-FITC/CD45RA-AF700	52.0 $\pm$ 1.6	67.0 $\pm$ 1.6	T: <0.0001

PBMC = peripheral blood mononuclear cells, T = time.

<sup>1</sup>PBMC were cultured for 6 d in RPMI-1640 with 10% autologous serum, PHA-L (10  $\mu$ g/mL), IL-2 (1 ng/mL), and IL-15 (1 ng/mL). Numbers for each phenotype are expressed as a percentage of the  $\gamma\delta$ TCR+ cell population.

<sup>2</sup>Subjects consumed either one (5 g) or 2 (10 g) servings of dried, prepared mushrooms daily for 4 weeks. Data from both groups were combined for analysis.

<sup>3</sup>Mean  $\pm$  SEM.

## Shiitake Mushrooms Improve Human Immunity

**Table 5.** Biomarkers of Immunity Detected in Saliva and Serum before and after Consumption of *L. edodes* Mushrooms<sup>1</sup>

		Baseline	4 Weeks	<i>p</i> Value
sIgA <sup>2</sup>	( $\mu$ g/ml)	815.2 $\pm$ 49.3 <sup>4</sup>	910.7 $\pm$ 81.0	0.049
sIgA	( $\mu$ g/min)	779.1 $\pm$ 58.2	917.9 $\pm$ 94.0	0.045
CRP <sup>3</sup>	CRP <sup>3</sup> (mg/L)	1.09 $\pm$ 0.72	0.77 $\pm$ 0.36	0.008

sIgA = secretory immunoglobulin A, CRP = C-reactive protein.

<sup>1</sup>Subjects consumed either one (5 g) or 2 (10 g) servings of dried, prepared mushrooms daily for 4 weeks. Data from both groups were combined for analysis.

<sup>2</sup>sIgA in the saliva was detected by ELISA and is expressed as both concentration ( $\mu$ g/ml) and rate ( $\mu$ g/min).

<sup>3</sup>Detection of CRP in the serum was performed using ELISA.

<sup>4</sup>Mean  $\pm$  SEM. A general linear mixed model that included time and treatment as fixed effects.

**Table 6.** Cytokine Production by Stimulated PBMC<sup>1</sup> before and after *L. edodes* Consumption<sup>2</sup>

Cytokine	Baseline	4 Weeks	<i>p</i> Value
IL-4	73.5 $\pm$ 9.63 <sup>3</sup>	111.3 $\pm$ 11.7	T: <0.0001, S: 0.045
IL-10	1962.7 $\pm$ 166.6	2618.2 $\pm$ 225.0	T: 0.0001
IL-1 $\alpha$	499.1 $\pm$ 40.0	581.7 $\pm$ 45.5	T: 0.0069
TNF- $\alpha$	3029.7 $\pm$ 185.6	3561.3 $\pm$ 243.5	T: 0.0032
MIP1- $\alpha$	26,953.8 $\pm$ 1276.3	8793.2 $\pm$ 176.6	T: <0.0001
IL-1 $\beta$	6103.4 $\pm$ 521.6	6107.2 $\pm$ 704.1	T: NS
IL-6	7943.2 $\pm$ 696.7	6047.2 $\pm$ 411.6	T: NS
IL-17	54.9 $\pm$ 6.4	60.7 $\pm$ 7.6	T: NS
IFN- $\gamma$	2282.1 $\pm$ 214.7	2071.7 $\pm$ 196.8	T: NS
MIP1- $\beta$	9622.8 $\pm$ 644.1	9645.7 $\pm$ 511.4	T: NS

PBMC = peripheral blood mononuclear cells, IL = interleukin, TNF = tumor necrosis factor, MIP = macrophage inflammatory protein, IFN = interferon, T = main effect of time, S = serving size covariate.

<sup>1</sup>PBMC were cultured for 24 hours in RPMI-1640 with 10% autologous serum, PHA-L (10  $\mu$ g/mL), IL-2 (1 ng/mL), and IL-15 (1 ng/mL).

<sup>2</sup>Subjects consumed either one (5 g) or 2 (10 g) servings of dried, prepared mushrooms daily for 4 weeks. Data from both groups were combined for analysis.

<sup>3</sup>Mean  $\pm$  SEM. Significance was determined by a general linear mixed model that included time period and treatment as fixed effects and random effect of subject to capture the repeated measurements on the same subject. Additional covariates included age, gender, and BMI and all 2-way interactions of these with treatment and time.

Serving size did not influence cytokine secretion except in the case of IL-4, where 2 servings of mushrooms were associated with higher levels than one serving ( $p = 0.045$ ).

## DISCUSSION

The literature suggests that consuming mushrooms will improve immunity, yet few studies in humans have been done. This clinical study fed whole mushrooms to humans as a food.

The dried *L. edodes* mushrooms we used required rehydration and heat processing before consumption. The 2 individuals in the one-serving group who dropped out of the study did so because they acquired shiitake dermatitis, which may have been due to improper preparation of the dried mushrooms before they were eaten. The number of subjects that dropped out of the 2-serving group, 10%, is the normal rate of attrition for our studies. The fact that it was due to gastrointestinal distress appears to be related to the consumption of the mushrooms; however, symptoms were not severe. In our analyses, the differences seen between the results for the 2 serving sizes tended to go in the right direction; that is, if the results for one serving were lower, then the results for two servings were even lower. However, only one measure, IL-4, actually achieved statistical significance. Another limitation of the study was that a placebo cannot be created when whole food is eaten. With no placebo, the study lacked a “no treatment” group. Had we observed a dose effect we would have been able to rule out changes due to time alone. The self-reported illness data we collected showed that the 2 groups had indistinguishable illness patterns; therefore, we feel that illnesses during the study did not have an effect on our results.

In our study, subjects consumed either 5 or 10 g of mushrooms daily for 4 weeks. In order to detect immune changes after mushroom consumption, we examined the proliferation and activation of 2 innate lymphocytes,  $\gamma\delta$ -T and NK-T cells. Before mushrooms were consumed, the proliferation and activation of these 2 cell types, in the presence of autologous serum and mitogen stimulation, was minimal. In contrast, after consumption these lymphocytes responded robustly to the mitogen stimulation.

The specific mechanism by which mushrooms alter a cell's ability to proliferate is not known. We hypothesize that components of the mushroom act to prime innate immune cells, thereby enhancing proliferation and activation responses of those cells upon stimulation. Priming itself does not evoke a cellular response but merely prepares the cell, enhancing its ability to respond to a secondary stimulus. We did find more naïve  $\gamma\delta$ -T cells in the peripheral blood after mushroom consumption, a cell type that is more likely to be primed than the memory cell.

Beta-glucans have been linked to immune alterations [16,19]. Lentinan, the specific  $\beta$ -1,3;1,6-glucan found in *L. edodes* mushrooms [19], is known to interact with both toll-like and dectin receptors [20–22] on macrophages, dendritic cells, and other immune cells. In the gut, the immune cells that sample lumen contents interact with the  $\beta$ -glucan and then, as these cells migrate into lymph and peripheral blood, they communicate their information to other immune cells. In addition, it is likely that  $\beta$ -glucan is fermented by microbes in the large intestine, and although it is known that compounds derived from this fermentation are absorbed, the exact mechanism by which they impact immune cells has not been determined. An

increase in the level of secretory IgA in the saliva is regarded as evidence of beneficial changes to mucosal immunity [23]. Our findings showed a rise in sIgA levels after consuming *L. edodes* mushroom, as has also been reported in humans using the white button (*A. bisporus*) mushroom [24].

There are many compounds in *L. edodes* mushrooms, besides  $\beta$ -glucan, that have the potential to influence immunity. For example, ergothioneine [25–27] and conjugated linolenic acid [28], as well as vitamins and minerals, are known bioactive compounds found in most mushrooms. In addition to priming, *L. edodes* mushroom consumption appeared to influence the immune response in such a way as to reduce inflammation. The immune response after priming is more vigorous, yet the cells produced fewer inflammatory mediators and more anti-inflammatory mediators. A more vigorous response, occurring under less detrimental conditions, would seem to favor the host organism.

CD69, a lectin receptor, is the earliest inducible cell surface glycoprotein acquired during lymphocyte activation [29]. Knock-out mice lacking CD69 are severely inflamed [30]. Our results showed that eating mushrooms increased the CD69 expression on the surface of  $\gamma\delta$ -T cells, which would suggest that there was a lower state of inflammation during the mitogen stimulation of cell proliferation.

Other evidence for this lower state of inflammation was seen in the cellular production of cytokines. The cytokines IL-4 and IL-10 are generally characterized as anti-inflammatory, because they downregulate the production of inflammatory cytokines. IL-4 is a regulatory cytokine that is responsible for the proliferation of B cells and Th2 helper cells. The main function of IL-10 is to terminate inflammatory responses by inhibiting the synthesis of IFN- $\gamma$  and TNF $\alpha$ . IL-4 and IL-10 were produced in greater quantity after the subjects consumed mushrooms. MIP-1 $\alpha$ /CCL3 is a chemotactic inflammatory cytokine produced early in an immune response. It induces the production of IL-1, IL-6, and TNF- $\alpha$ . Of all of the cytokines we measured, this was the only one whose production decreased after mushroom consumption. Less MIP-1 $\alpha$ /CCL3 should inhibit the amount of IL-1, IL-6, and TNF- $\alpha$  produced. However, we saw TNF- $\alpha$  concentrations rise, while the cytokines IL-1 and IL-6 remained the same. An elevated TNF- $\alpha$  concentration has been reported in other *in vitro* [5–7] and *in vivo* [12,13] studies. TNF- $\alpha$  is often classified as an inflammatory cytokine, although one of its functions is to resolve inflammation by causing apoptosis. The fact that TNF- $\alpha$  levels increased, despite the increase in IL-10 and decrease in MIP-1 $\alpha$ /CCL3, suggests that we still do not know all there is to know about the regulation of this cytokine.

CRP, the prototypical acute phase protein in humans, participates in the nonspecific systemic response and its levels rise in response to inflammation [31]. The fact that the final concentration of CRP was significantly reduced in the subjects consuming mushrooms, who started with values within the

normal range for that population, again suggests that eating mushrooms lowers inflammation.

Changes we saw in the NK-T cells paralleled those of the  $\gamma\delta$ -T cells, suggesting that other innate lymphocytes may be primed by dietary compounds. NKG2D (CD314) is a c-type lectin receptor that is not highly expressed on the surface of cells under normal resting conditions. At 4 weeks, NKG2D expression on resting, uncultured cells was significantly lower than that seen before mushroom consumption. This lowering of the resting activation state suggests reduced inflammation.

The expression of NKG2D increases on the cell surface when cells are activated [32,33]. In this study, mitogen-stimulated NK-T cells expressed greater than 10-fold more NKG2D on the cell surface after mushroom consumption than cells stimulated before consumption. An increase in NKG2D expression may be a part of the priming mechanism, as we have suggested for CD69 expression on  $\gamma\delta$ -T cells. NKG2D is part of the machinery used by NK cells to lyse infected or tumor cells [32]. Increased expression of this molecule on the cell surface suggests an improvement in the cytolytic ability of NK-T cells, which may contribute to the resolution of the immune response by causing apoptosis of cells that are no longer required when the pathogen threat is eliminated.

In conclusion, we believe that regular *L. edodes* mushroom consumption resulted in improved immunity, as seen by increased cellular proliferation and activation after stimulation and the higher levels of sIgA produced. We suggest that the mechanism by which this happens is the priming of innate lymphocytes by *L. edodes*, making them able to respond more robustly to a challenge. In addition, we feel that the expression of activation markers CD69 and NKG2D on innate T cells, and the pattern of cytokines secreted by the PBMC, along with the reduction in the level of CRP, together suggest the presence of an anti-inflammatory environment during stimulation by the mitogen. It seems likely that less inflammation during an immune response may be beneficial to the host; this results in a less aggressive immune response, while the ability of the organism to energetically combat a pathogen is preserved.

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## Authors' Contributions

X.D., J.M.S., B.L.-H., and S.S.P. designed the research. X.D., J.M.S., C.A.R., E.A.S., C.N., and S.J.S. conducted the research. X.D. and J.M.S. analyzed the data, and M.C.C. performed the statistical analysis. X.D., J.M.S., C.A.R., B.L.-H., and S.S.P. wrote and edited the article. X.D. and J.M.S. contributed equally to the work in this manuscript. S.S.P. had primary responsibility for final content. All authors read and approved the final article. None of the authors had any conflicts of interest.

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