Dietary Supplementation with White Button Mushrooms Augments the Protective Immune Response to *Salmonella* Vaccine in Mice\(^{1,2}\)

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**Abstract**

We previously showed that dietary white button mushrooms (WBMs) enhanced natural killer cell activity and that in vitro WBM supplementation promotes maturation and function of dendritic cells (DCs). The current study investigated whether WBM consumption would enhance pathogen-specific immune response using a *Salmonella* vaccination and infection animal model. C57BL/6 mice were fed diets containing 0%, 2%, or 5% WBM for 4 wk before oral vaccination with live attenuated *Salmonella typhimurium* SL1479. Four weeks after immunization, mice were orally infected with virulent *Salmonella typhimurium* SL1344. Immunization increased animal survival and, among immunized mice, the 2% WBM group had a higher survival rate than the other groups. Next, we fed mice 2% WBMs to determine the immunological mechanism underlying the WBM-potentiated protective effect. We found that WBM supplementation increased *Salmonella*-specific blood immunoglobulin (Ig) G and fecal IgA concentrations. WBM-fed mice also had a higher IgG2a and unchanged IgG1 production, leading to an elevated IgG2a:IgG1 ratio and indicating an enhanced T helper 1 response. Consistent with these results, WBM-fed mice had higher interferon-\(\gamma\) (IFN-\(\gamma\)), tumor necrosis factor (TNF)-\(\alpha\), and interleukin (IL)-17A production and unchanged IL-4 production in their splenocytes after polyclonal (anti-CD3/CD28) or antigen-specific stimulation. Furthermore, WBM-fed mice had more DCs in the spleen, and these DCs expressed higher levels of activation markers CD40 and major histocompatibility complex II. These mice also produced more IL-12 and TNF-\(\alpha\) postimmunization. Together, these results suggest that WBMs may improve *Salmonella* vaccine efficacy through an enhanced adaptive immune response. J. Nutr. 144: 98–105, 2014.

**Introduction**

*Salmonella enterica* is a Gram-negative, facultative, intracellular pathogen that is transmitted through contaminated food and water and can result in infections in humans and animals. *Salmonella* infection is pandemic worldwide, particularly in developing countries (1). In industrialized countries, *Salmonella* is among the most frequently isolated species of foodborne infections. In the United States, 30–50% of foodborne pathogen infections are attributed to *Salmonella* serovars, and *Salmonella* infection is the leading cause of foodborne fatalities (2–4). Different clinical outcomes of *Salmonella* infection, ranging from self-limiting gastroenteritis to a life-threatening systemic infection (typhoid fever), are primarily determined by the infecting *Salmonella* serovars, but they are also known to be related to the host’s susceptibility (5). *Salmonella* serovar Typhi causes human typhoid fever, which infects >21 million people and results in >200,000 deaths annually (6), whereas *Salmonella* serovar Typhimurium (*S. typhimurium*) causes a self-limiting gastroenteritis in humans but can result in fatal infections in hosts with compromised immunity (7). *S. typhimurium* causes a systemic typhoid-like disease in mice, which is why the mouse *S. typhimurium* infection is a widely used model system for the study of host immunity against systemic *Salmonella* infection (S. Typhi) (8,9).

Vaccination is an effective means for preventing *Salmonella* infection (10,11). Much of the knowledge about developing protective immunity against *Salmonella* infection is from the studies using animal models (12,13). Protection, which requires both cellular and humoral immunity, has been achieved by immunizing susceptible mice with live vaccine strains (14–17). Although there are still no licensed vaccines available for human nontyphoidal *S. typhimurium* infection (18), currently there are 2 vaccines approved for typhoid fever (10,11,19). Although the WHO recommends vaccination of travelers or residents in...
Mushrooms, immunity, and Salmonella vaccination

Materials and Methods

Mice and diets. Specific pathogen-free female C57BL/6 mice (6–8 wk) were purchased from Charles River Laboratories and individually housed in environmentally controlled cages with a 12-h-light/-dark cycle. Mice were provided with free access to water and the experimental diets. Body weight was recorded weekly before infection and daily after infection. All animal-related procedures were approved by the Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals. Fresh WBMs were provided by the Country Fresh Mushroom Co. through the Mushroom Council. The mushroom powder was prepared and added at 2% or 5% (wt/wt) to an AIN-93G diet (Research Diets) (2%WBM and 5%WBM, respectively) as previously described (24,25). These doses are considered translationally relevant, because they are achievable through normal dietary intake. The 2% dose for mice can be converted to a daily consumption of 2.2 g fresh mushrooms/kg body weight for humans by using isocaloric calculation (28), or ~150 g fresh mushrooms/d (2 servings) for a person weighing 65–70 kg. Similarly, the 5% mushroom is equivalent to 5 servings/d.

Bacterial strains. SL1344, a virulent S. typhimurium strain, was kindly provided as a gift by J. Mecsas (Tufts University). An attenuated strain, S. typhimurium SL1479 Aro A, was purchased from American Type Culture Collection and was used as Salmonella vaccine against SL1344 infection challenge as previously reported (29). The bacteria SL1344 and SL1479 were grown overnight with aeration in Luria-Bertani and Tryptic Soy broth, respectively. The bacteria were washed and resuspended with cold PBS to the appropriate concentrations for oral inoculation of mice.

Preparation of S. typhimurium antigen. After S. typhimurium SL1479 bacteria were cultured overnight, they were centrifuged and washed with and resuspended in cold PBS. To prepare the heating-killed S. typhimurium antigen, the bacteria in suspension were incubated at 60°C in a water bath for 60 min. This preparation was used as antigen for ex vivo stimulation of splenocytes. The sonication-killed S. typhimurium was prepared as previously described (30) and was used as coating antigen in ELISA. The concentrations of bacteria were estimated by quantification of total protein with BCA protein assay (Pierce Biotechnology).

Salmonella immunization and infection. This study was divided into 2 experiments (Fig. 1). In Expt. 1, we determined the effect of WBM supplementation on Salmonella infection in mice with or without a previous vaccination. In Expt. 2, we determined the effect of WBM supplementation on the relevant immune responses after Salmonella vaccination. In Expt. 1, mice were divided into 4 groups (n = 16/group). The control and control plus vaccine (C+V) groups were fed the control diet and the 2%WBM plus vaccine (2%WBM+V) and 5%WBM plus vaccine (5%WBM+V) groups were fed 2% and 5%WBM, respectively. After 4 wk of feeding, mice in all groups except the control group were

Experiment 1: Vaccine protection against Salmonella infection

Experiment 2: Evaluation of immune response to vaccine

FIGURE 1 Study design. The attenuated S. typhimurium SL1479 was used as vaccine and the virulent wild-type S. typhimurium SL1344 was used for infection. n = 16/group in Expt. 1, n = 12/group in Expt. 2. 2%WBM, control diet plus 2% white button mushroom powder; 5%WBM, control diet plus 5% white button mushroom powder.
immunized by oral administration of $3 \times 10^7$ CFU of attenuated S. typhimurium SL1479 and mice in the control group were sham-inoculated with PBS as control. At 4 wk postimmunization, all mice were orally infected with $1 \times 10^7$ CFU of virulent S. typhimurium SL1344. Body weight was recorded daily. Mortality was based on actual death or when weight loss was $>15\%$ of their initial (pre-infection) weight. In Expt. 2, mice were divided into 4 groups as defined above ($n = 12$/group). The control and C+V groups were fed the control diet and the 2%WBM and 2%WBM+V groups were fed 2% WBM. After 4 wk of feeding, mice in the C+V and 2%WBM+V groups were immunized by oral administration of S. typhimurium SL1479 and mice in the control and 2%WBM groups were sham-inoculated with PBS as control. At 4 wk postimmunization, fresh feces were collected and all mice were killed by CO2 inhalation followed by blood and spleen collection for immunological analysis.

**Determination of antibody titers and IgG isotyping.** IgG and IgA concentrations were determined in serum and fecal samples, respectively. Fecal samples were prepared and total IgA in extracted fecal samples as well as total IgG in serum samples were measured as previously described (29,30). Salmonella-specific IgA, IgG, and IgG isotypes were determined using the same ELISA procedure except that the plates were coated with sonication-killed S. typhimurium.

**Determination of cytokines.** Four weeks postimmunization, a single-cell suspension was prepared from spleen and the resulting splenocytes were cultured in 24-well plates in the presence of precoated anti-CD3 and soluble anti-CD28 or heating-killed S. typhimurium for 72 h to determine the production of T-cell cytokines. Another set of splenocytes was cultured in the presence of LPS for 24 h for production of inflammatory cytokines. Cell-free supernatants were collected at the end of incubation and stored until analysis. The quantification of IL-4, IL-6, IL-10, IL-17A, IFN-γ, and tumor necrosis factor (TNF)-α in the supernatants was conducted using the mouse cytometric bead array kit according to the manufacturer’s instructions (CBA; BD Biosciences). The fluorescence signals associated with cytokine-bead complex were acquired using a BD Accuri C6 flow cytometer and data were analyzed using Flowjo 7.6 software (Tree Star).

**Statistical analysis.** Survival analysis was conducted using the Kaplan-Meier method and comparisons were made between groups using the log-rank test (GraphPad Prism Software). All the other data were analyzed using 2-factor ANOVA in the SYSTAT 12 statistical software. When a significant interaction between vaccine and diet was found, a Bonferroni post hoc test was conducted for pair-wise comparisons of diet effect. Significance was set at $P < 0.05$.

**Results**

**WBM supplementation further enhances the protective effect of vaccine against Salmonella challenge.** Mice immunized with attenuated S. typhimurium SL1479 had a late onset of mortality and a higher survival rate compared with the control (nonimmunized) mice; this improved survival rate was further enhanced in the 2%WBM+V mice relative to the C+V mice (Fig. 2). However, the 5%WBM+V mice had a survival rate that did not differ from the C+V mice. Because the 2% but not 5%WBM-fed mice had a potentiated protection after Salmonella vaccination, we used this supplementation amount in the subsequent study to further investigate the immunological mechanisms underlying the improved protection.

**WBM supplementation enhances antibody production induced by Salmonella vaccination.** Compared with the nonimmunized groups, vaccination induced production of both total and Salmonella-specific IgG in serum and slgA in fecal pellets (Fig. 3). Although the diet did not affect total IgG
production (Fig. 3A), WBM supplementation resulted in a higher production of Salmonella-specific IgG (Fig. 3C). In contrast, both total and Salmonella-specific IgA were higher in mice fed WBM compared with those fed the control diet (Fig. 3B,D).

**WBM supplementation induces increased production of IgG2a but has no effect on IgG1, leading to higher IgG2a:IgG1 ratio.** Because the IgG isotypes IgG2a and IgG1 are related to the antigen-specific T-cell response pattern T helper (Th) 1 and Th2, respectively, we further determined serum concentrations of Salmonella-specific IgG2a and IgG1. Compared with vaccination, which greatly induced Salmonella-specific IgG2a and IgG1 responses, WBM supplementation resulted in a greater increase in IgG2a but had no effect on IgG1 production (Fig. 4A, B). As a result, WBM supplementation greatly increased the IgG2a:IgG1 ratio (Fig. 4C), which suggests a Th1 polarized response.

**WBM supplementation promotes Th1 and Th17 response.** To assess the relative contribution of different components of T-cell response in WBM-induced enhancement of vaccination efficacy, we determined the production of indicator cytokines appropriate for the corresponding T-cell populations, Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-10), and Th17 (IL-17A), under conditions of polyclonal (T-cell receptor Ab) or antigen (Salmonella typhimurium) stimulation ex vivo. Under the former condition, while all of the above-mentioned cytokines were upregulated in response to the vaccination, WBM induction potentiated production of IFN-γ, TNF-α, and IL-17A but had no effect on IL-4 and prevented induction of IL-10 (Fig. 5). Similar induction responding to vaccination was found in Salmonella-specific IFN-γ, TNF-α, IL-17A, and IL-10 production (Fig. 6). WBM supplementation potentiated production of all the cytokines in immunized mice except for IL-10, which was unaffected (Fig. 6D). Salmonella-specific IL-4 production was undetectable.

**WBM supplementation increases the frequency and maturation of DCs in spleen.** There was an overall effect of both diet and vaccine on the frequency of DCs (CD11c+ cells) and their activation markers MHC-II and CD40 as well as a significant diet × vaccine interaction in MHC-II expression (Fig. 7). We also measured another DC activation marker, CD86, and found no change related to either vaccination or diet (data not shown).

**Effect of Salmonella vaccine and WBM supplementation on production of proinflammatory cytokines.** Cytokines are involved in DC’s antigen-presenting function as well as the development of T-cell effector response. We measured some of these cytokines involved in the immune response to Salmonella and found that splenocyte production of IL-12, TNF-α, and IL-6, but not IL-1β, was higher in immunized mice than in nonimmunized mice when splenocytes were stimulated ex vivo with LPS (Fig. 8). Furthermore, upregulated IL-12 and TNF-α production by Salmonella vaccination was further potentiated by WBM supplementation (Fig. 8A, B).
The importance of both CD4+ T cells and B cells in developing protection against humoral and cellular immunity are required for efficient host adjuvant for vaccines against foodborne pathogen infections. Candidate to serve as a food-based, safe, and effective mucosal strain. These data suggest that WBMs may be a good reduced mortality when mice are challenged with a virulent cellular and humoral responses against the vaccine as well as by vaccine

**Discussion**

The efficacy of oral immunization is often reduced due to a number of factors such as instability of antigens in the gastrointestinal tract, weak antigen uptake from mucosal surfaces, and impaired induction of adaptive immune response (31,32). Effective mucosal adjuvants may help induce robust mucosal and systemic immune responses. Evidence from both animal and human studies suggests that nutritional intervention may help improve vaccine efficacy by favorably modulating the immune responses involved in the development of adaptive immunity (30,33–35). In this study, we investigated whether dietary intake of WBMs could increase vaccine efficacy against Salmonella by using an attenuated S. typhimurium vaccine. Our results demonstrate that WBM supplementation enhances the Salmonella vaccine’s efficacy as evidenced by enhancement in both cellular and humoral responses against the vaccine as well as by reduced mortality when mice are challenged with a virulent Salmonella strain. These data suggest that WBMs may be a good candidate to serve as a food-based, safe, and effective mucosal adjuvant for vaccines against foodborne pathogen infections.

As in the case of other facultative intracellular bacteria, both humoral and cellular immunity are required for efficient host protection against S. typhimurium (36). Studies have shown the importance of both CD4+ T cells and B cells in developing protective immunity against Salmonella after initial exposure (infection or vaccination) (37,38). Although the cellular immune response is recognized to be crucial for protective immunity against an intracellular pathogen like Salmonella, the pathogen-specific B-cell response also contributes to bacterial clearance, mainly by producing antibodies (16,39). sIgA, the primary mucosal antibody class produced by local plasma cells, can block secondary Salmonella infection in the intestinal lumen by inhibiting bacterial adherence and invasion of epithelial and M cells (16,40). Systemic antibodies (IgG and IgA) help eliminate Salmonella from blood and invaded organs by providing opsonization of bacteria, which promotes their phagocytosis (41). In the current study, dietary WBMs induced a higher production of total and Salmonella-specific IgA in the immunized mice but had no such effect in the nonimmunized mice. This result is in contrast to Jeong et al. (26), who reported that consumption of WBMs by healthy human volunteers resulted in enhanced total salivary IgA secretion lasting for only 2 wk. Their results may represent a transient, nonspecific, stimulating effect of WBMs on the gut mucosal immune system rather than a manifestation of enhanced, sustained, specific immunity as observed in our study. The difference in the participants studied, WBM preparation, administration, and dosage might all have contributed to the difference in the results reported by the 2 studies. We also found that the vaccination induced higher concentrations of both total and Salmonella-specific IgG in serum, and WBM supplementation potentiated Salmonella-specific IgG but not total IgG. Together, these results suggest that WBMs may serve as a mucosal adjuvant to enhance vaccine-induced mucosal and systemic humoral immunity, resulting in further improved host protection.

\[ \text{T-cell-mediated immune response is indispensable for intracellular pathogen infections, including those caused by Salmonella. Salmonella-specific CD4+ T cells, in particular the Th1 cells that produce IFN-\gamma, are thought to be a key player in mediating the protective effect of Salmonella vaccination (41). In this study, we found that WBM supplementation induced a further increase in the production of Th1 cytokines IFN-\gamma and TNF-\alpha in immunized mice, which suggests an enhanced Th1 response. This assessment was further supported by the increased IL-12 production observed in WBM-fed mice, because Th1 polarization is mainly driven by IL-12 (42,43). IL-12, which can be induced during Salmonella infection (44), contributes to the host's defense against Salmonella, an effect likely mediated by IFN-\gamma (45). These results are consistent with the WBM-induced change in IgG isotype, i.e., higher IgG2a and IgG2a: IgG1 ratio, given that Th1 cells direct cell-mediated immunity and promote a class switch to IgG2a while Th2 cells direct humoral immunity and promote a class switch to IgG1 (46,47).} \]
Likewise, unaltered production of Th2 cytokine IL-4 with WBM supplementation also echoed the lack of change in IgG1 production. IL-4–producing Th2 cells are known to compromise protective immunity against Salmonella (44, 48). Although IL-10 is also viewed as a Th2 cytokine, its production is mainly attributed to activated monocytes/macrophages and another type of CD4+ T cells, Treg cells. We found that WBM supplementation did not affect Salmonella-specific IL-10 production but caused a decrease in IL-10 production induced by polyclonal T-cell stimulation. Because IL-10 is an anti-inflammatory cytokine, its decreased production together with the increased production of proinflammatory cytokines (IFN-γ, TNF-α, IL-12, IL-17) seems to be in line with an enhanced inflammatory state. As such, the overall change is expected to favor antigen presentation and T-cell effector function. IL-17–producing Th17 cells are a recently defined subtype of CD4+ T cells and it has been suggested that these cells play a protective role against Salmonella infection (41). Th17 cytokines have been shown to induce production of antimicrobial peptides against luminal bacteria by epithelial cells (49). The observed increase in IL-17 production in WBM-fed mice may reflect an increase in Th17 population. Together, these results suggest that WSBMs induce Th1 and Th17 dominant responses while reducing or having little effect on Th2 response, which is expected to contribute to the observed enhancement in anti-Salmonella immunity.

Initiation of adaptive immunity against bacteria requires APCs, which recognize, process, and present bacterial antigens to naïve T cells. DCs, the most important professional APCs, play a critical role in initiating antibacterial adaptive immunity (50–53). After pathogen-associated molecular pattern detection at the infection site occurs, immature DCs become activated and undergo maturation as characterized by phenotypic change (53, 54). This maturation is necessary for DCs to efficiently present an antigen to naïve T cells, leading to the development of adaptive immunity. DCs can respond to Salmonella's LPS, or flagellin, by increasing expression of MHC-II and the co-stimulatory molecules CD80, CD86, and CD40 (55, 56). In vitro addition of polysaccharides isolated from mushrooms (20–22) or WBMs (23) enhances DC maturation and function. The physiological relevance of these in vitro observations is supported by the data presented in the current study using an oral Salmonella vaccination model, because we found increased DC frequency and increased expression of MHC-II and CD40 with dietary WBM supplementation. The increased production of IL-12 and Th1 cytokines seen in WBM-fed mice may also reflect the upregulated DC numbers and their function, because DCs are the main source of IL-12 and IL-12 drives Th1 polarization (42, 43).

In the infection-survival study, we used 2 amounts of WBM supplementation and expected to observe that WBM's effect would be dose dependent. However, it is interesting to note that mice fed 2% WBM had a significantly enhanced survival compared with vaccine alone, whereas those fed 5%WBM did not. A possible explanation is that a higher dose of WBMs may cause more weight loss through increasing the proinflammatory cytokine production under the infection condition. Infection can induce production of proinflammatory cytokines. These cytokines can promote maturation and activity of innate immune cells and also facilitate development of adaptive immunity. On the other hand, however, these cytokines may contribute to tissue damage and clinical symptoms (such as weight loss) in infection. In a previous study, we found that mice fed 10% WBM compared with 2% WBM had higher production of proinflammatory cytokines IL-1β and TNF-α at day 5 and tended to have more weight loss at day 6 after influenza infection (25). Because we did not collect samples to determine the cytokine production in the infection-survival study (Expt. 1), we can only speculate that lower survival (>15% weight loss counted as mortality) in the 5%WBM compared with the 2% WBM group might reflect more weight loss associated with higher production of proinflammatory cytokines.

In summary, we have shown that dietary WBM supplementation potentiates the efficacy of Salmonella vaccination and results in better protection against subsequent exposure to salmonella infection. This improved vaccination efficacy is associated with elevated immune response as shown by enhancement in both cellular and humoral responses as well as by DC activation. These results suggest that WBM intake could be considered as a strategy to optimize the efficacy of some vaccines. Future studies are needed to determine the impact of WBMs on other vaccines and to validate the clinical relevance of current findings in humans.

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Literature Cited

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