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Maitake mushrooms (*Grifola frondosa*) enhances antibody production in response to influenza vaccination in healthy adult volunteers concurrent with alleviation of common cold symptoms

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ABSTRACT

Background: The prevention of influenza virus infection is a critical public health challenge.Thus new, safe, and effective strategies are needed to reduce the risk of infection. Maitake mushrooms (*Grifola frondosa*) are popular in Asia for their flavor and immune-stimulating properties. In this study, we examined whether the dietary consumption of Maitake was effective in boosting the potency of influenza virus vaccination.

Methods: We set up a double-blind, placebo-controlled clinical trial (n=50 subjects for Maitake consumption; n=50 for placebo) and analyzed hemagglutination inhibition (HI) antibody titers in

response to trivalent influenza vaccine (type A H1N1, H3N2, and type B BX-51B) for a 12-week period of daily Maitake intake (6.825g), beginning 4 weeks before and continuing 8 weeks after vaccination. We also evaluated the efficacy of Maitake for suppression of common cold symptoms by questionnaire.

Results: We found that continuous Maitake intake raised HI titers against influenza type A virus H1N1 and type B virus, and significantly increased the seroconversion rate for older adults (>60 years of age). Additionally, severe cold symptoms including rhinorrhea and headache were significantly improved by Maitake intake.

Conclusion: In this clinical trial, we demonstrated that Maitake intake enhanced antibody production in response to influenza vaccination while simultaneously suppressing multiple common cold symptoms. The current results suggest that Maitake may activate both innate and adaptive immune responses for the prevention of virus infection. In conclusion, we expect that Maitake intake potentiates host defense systems and has a protective effect against influenza virus and other pathogenic viruses and bacteria.

Keywords: clinical trial, *Grifola frondosa*, influenza, Maitake, vaccine

INTRODUCTION

Influenza virus is a major cause of respiratory tract infection; it affects all age groups and can recur in any affected individual [1]. Influenza epidemics occur almost every winter, causing substantial social and economic damage in addition to severe illness and death. The prevention of influenza virus infections is an important public health challenge. Effective therapies to prevent and treat influenza virus infections could reduce morbidity and economic losses resulting from this illness, and are thereby in high demand [2, 3]. Vaccination, which induces adaptive immune mechanisms such as antigen-specific antibody and cytotoxic T cells is by far the most effective procedure to prevent influenza virus infections. Influenza vaccination is recommended not only to protect against influenza virus infection in particular pandemic infection but to also reduce the risk of severe diseases such as influenza encephalitis and pneumonia [4].

Influenza can be prevented and is certainly well managed by vaccinations that are technically available for use even in developing countries [4]. However, vaccination is not an absolute solution to prevent viral infection. For example, influenza virus infections, particularly pandemic avian influenza A/H5N1, are especially dangerous to specific populations such as older adults, diabetes patients, infants, and pregnant women due to lowered or poor response to vaccination [5, 6, 7]. According to several reports, immune responses are weakened by specific lifestyle attributes, such as obesity [8], mental and physical stress [9, 10], and smoking cigarettes [11]. Therefore, boosting

and maintaining robust innate and adaptive immunity is critical for the prevention of the severe health impacts of influenza. In this context, effective methods to improve the efficacy of vaccination are being sought.

β-glucan, the main component of the cell walls of mushrooms, has been well demonstrated to have the effect of enhancing the immune system [12]. A series of reports have been published on the effects on the immune system of heteropolysaccharides and the low-molecular-weight protein fraction (D-fraction) extracted from Maitake mushrooms [13-19]. The D-fraction is effective for suppression of tumor growth and viral/bacterial infection through activation of immunocompetent cells. These polysaccharides enhance the activities of immune potent cells such as macrophages, helper T cells, and cytotoxic T cells.

In addition to their effects on the immune system, dietary mushrooms have been demonstrated to improve insulin resistance in diabetes [20, 21] and to have cholesterol-lowering [22], anti-atherogenic [23], anti-hypertensive [24], inflammatory bowel disease-inhibitory [25], and anti-allergic [26] effects. The general impact of this extensive body of research is that Maitake is widely accepted to have physiological effects promoting the maintenance and improvement of health.

Recently, deoxyribonucleic acid (DNA) microarray analysis demonstrated that the dietary consumption of Maitake mushrooms, which are a common and traditional food in Japan, was effective for upregulation of the gene involved in the signal transduction of innate immunity via toll-like receptor (TLR) 3 and interferon, as well as several virus-resistance genes [27]. Moreover, the microarray analysis data indicated that Maitake mushrooms would help prevent influenza virus infection. To examine this hypothesis, we performed a human clinical trial to investigate the immune function of Maitake mushrooms, focusing on the effect of 12-week intake on the potency of influenza vaccine. In parallel, we evaluated the effects of Maitake mushrooms on innate immune function by assessing the common cold symptoms of subjects using questionnaires.

SUBJECTS AND METHODS

Subjects

We recruited healthy Japanese men and women between the ages of 30 and 70 years who were living in Ebetsu City and neighboring communities in Hokkaido, Japan. Volunteers who met any of the following criteria were excluded from participation in this study: 1) frequent intake (more than 5 days a week) of any type of mushrooms, 2) any current relevant infections such as influenza, 3) vaccination against influenza virus within 12 weeks before study entry, 4) current use of immunomodulating medicines (antibiotics, immunosuppressive medicine, anti-inflammatory medicine), Chinese herbs, supplements (such as mushroom, yeast, lactic acid bacteria preparation, or seaweed), 5) current use of any medicine for diarrhea or constipation, 6) history of significant illness, 7) pregnancy or lactation, 8) heavy smoking (more than 20 cigarettes/day) and/or excessive

alcohol consumption (more than 20 g alcohol/day), and 9) history of severe allergic reaction to food, medication, and vaccine. Participants were asked to terminate the intake of any foods or supplements containing mushrooms.

The sample size was statistically determined to obtain a power of 80% with an alpha value if 0.05%. We calculated the sample size with reference to data from other studies of mushroom extract administered as immune adjuvants [28]. In order to demonstrate an effect in the HI antibody titers against BX-51B at 12 weeks after the study, which was postulated to be a 10.00 increase with a standard deviation of 15.70, a sample size of 80 (40 in the active test food group and 40 in the placebo food group) was required. Assuming 20 loss during follow-up, we set the number of subjects to 50 in each group.

Test Samples

Maitake mushroom (strain: Gf433, variety denomination: Taisetsu hananomai No.1) was cultured in Forest Products Research Institute, Asahikawa (Hokkaido, Japan) as described in Yoneyama et al [29]. The harvested fruit-body was autoclaved at 100 °C for 10 min, freeze-dried, powdered, and prepared in the form of tablets. Two types of test sample were prepared, Maitake mushroom and a non-active placebo, which was mostly made of dextrin.

Study Design

The intervention was performed in a placebo-controlled, randomized, double-blind clinical trial. Subjects were randomly divided into two groups, an active Maitake group (n=50) and a control (placebo) group (n=50). All of the subjects consumed 6.825g/day (20 tablets/day) of either active Maitake mushrooms or placebo for 12 weeks. The daily dose was determined in terms of safety and efficacy according to the previous mushroom study [30]. We advised the volunteers not to change their daily lifestyle, including meals, exercise, and alcohol consumption during this study. A blood sample was collected at week 0 (W0) before intake of the first test sample.

A number of variants of the influenza viruses co-circulate each year. There is little cross-immunity between influenza types/subtypes or lineages. This is why several influenza strains must be included into combination vaccines [31]. After a pre-vaccination period of 4 weeks during which subjects were consuming their respective test samples, all subjects were administered a trivalent inactivated influenza vaccine (TIV) consisting of A/California/7/2009(X-179A) (H1N1) pdm09, A/New York/39/2012(X-233A) (H3N2), and B/Massachusetts/2/2012(BX-51B)). Blood samples were collected at week 4 (the time of vaccination), and weeks 8 and 12. All blood samples were analyzed for the hemagglutination inhibition (HI) antibody titer, and immunogenic biomarkers of blood samples at weeks 0, 4, 8, and 12 were analyzed.

Assessment of Immunogenicity

HI tests were performed using hemagglutinating antigens and reference antisera for influenza virus types A and B (Denka Seiken, Japan) according to standard procedures at a clinical laboratory testing company (SRL, Japan). Immunogenicity was evaluated as the geometric mean HI antibody titer (fold rise) to each of the three influenza antigens. The seroconversion rate was defined as ≥1:40 HI titer at post-vaccination for subjects with less than 10-fold at the baseline, or the proportion of subjects with a ≥ 4-fold increase in HI titers, in which seroprotection rate of > 40% for subjects 18–60 years old and that of >30% for those over 60 years. The seroprotection rate was defined as >70% for those 18–60 years old and >60% for those > 60 years old in cases with HI titers ≥1:40 [31]. These biomarkers were measured at a clinical laboratory testing center (Sapporo Clinical Laboratory Inc., Sapporo, Japan).

As for natural killer (NK) cell activity, Chromium-51 (^{51}Cr) release assays were used to measure NK cell activity (PerkinElmer, Waltham, MA) at weeks 0, 8, and 12. Target cells were labeled with ^{51}Cr, and the label was then released from the target cells by cytolysis. The supernatants from the centrifugation could be counted directly by a gamma counter. All measurements were performed by the SRL clinical laboratory (Tokyo, Japan).

Serum Biochemical Measurement

Hematological analyses were carried out with all blood samples [white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), and platelet count (Plt)]. Serum biochemical analyses were carried out with blood samples of weeks 0, 8, and 12, including total immunoglobulin G (IgG), total immunoglobulin A (IgA), liver function test [aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH)], renal function test [blood urea nitrogen (BUN), creatinine (CRE), and ureic acid (UA)], serum lipids [total cholesterol (T-Cho), low density lipoprotein cholesterol (LDL-Cho), high density lipoprotein cholesterol (HDL-Cho), and Triglyceride (TG)], C-reactive protein (CRP), and blood glucose. These biomarkers were measured at a clinical laboratory testing center (Sapporo Clinical Laboratory Inc., Sapporo, Japan).

Questionnaire on Common Cold Symptoms

All subjects were asked to report daily on any common cold symptoms (headache, muscle pain and arthralgia, general malaise, fever, chill, cough, phlegm, sore throat, runny nose, stuffy nose, loss of appetite, vomiting, sneezing, and diarrhea) experienced during the study. For each symptom, levels of severity were set by degrees from 1 to 5 points, with 5 being most severe. Total numbers were summed up for analysis, and incidence rates (%) for each symptom were divided by the total

incidence for each symptom. In cases in which subjects were suspected to have been infected by influenza viruses rather than the common cold, they were immediately checked by a rapid influenza immunoassay kit and examined by physicians.

Statistical Analysis

All data were collected and analyzed independently of the investigators, who did not have access to the data or to its analysis. Only data analysts could access the data to perform statistical analyses. As for compliance on data, they were stored and maintained on secure servers, and backed up daily. Security updates were made in timely manner regularly to ensure appropriate protections of the database. The between-group comparisons of the geometric means of the HI titer were assessed by Student's *t*-test. Differences in the seroconversion rate and the seroprotection rate between the Maitake and placebo groups were assessed by the chi-square test. The interaction between the group and the period of consumption (group-by-time interaction) was analyzed using repeated-measures analysis of variance (ANOVA). The between-group comparisons of actual values and the amount of change from baseline were assessed by Student's *t*-test. Statistical analyses were performed using SPSS Statistic 20 (IBM, NY). A significance level of $p < 0.05$ was considered to indicate significance.

Ethics Committee

Ethical clearance was obtained from the ethics committee of the Hokkaido Information University before the study was initiated (certificate number: 2014-11). Informed consent was obtained after the contents of this study were explained. Any undesirable events affecting a subject's health during the course of the study were defined as adverse events. If a subject asked to be released from the study, the protocol was immediately terminated for that subject. This study was conducted in accordance with the Declaration of Helsinki. This study was registered at the UMIN Clinical Trial Registry (UMIN000015209) on September 19, 2014.

RESULTS

Baseline and demographic data

Participants were recruited from among the residents of Ebetsu City (Hokkaido, Japan) and neighboring communities. The flow of participant involvement through the trial is shown in Fig. 1. Subjects who provided consent (n=140) were assessed for eligibility, and a total of 100 subjects were enrolled in this study. All enrolled subjects were randomized to one of the two intervention groups (Maitake group, n=50; placebo group, n=50). During the course of this study, 6 subjects discontinued the trial for personal reasons, such as starting of medication or relocation of residence. The number of evaluable subjects was 94 (Maitake group, n=45; placebo group, n=49). Two

subjects were excluded from analysis due to poor compliance. Thus the final number of subjects for analysis was 92 (Maitake group, n=44; placebo group, n=48). Average ages were 51.7 and 55.5 years old for the placebo and Maitake groups, respectively.

We found no significant differences between the Maitake and placebo groups in regard to age, gender, body mass index (BMI), body fat, or intake rate of test foods (Table 1).

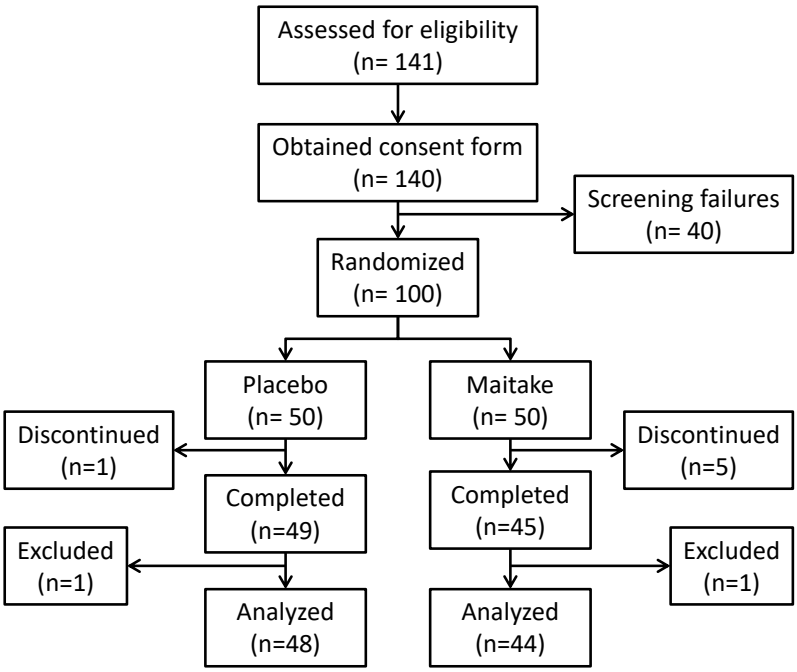

Figure 1. Subject characteristics. Values are expressed as the number of participants.

Table 1. Characteristics of the subjects in the placebo and Maitake groups at the screening test.

Characteristic	Placebo	Maitake	*P*-value
Subjects, n	48	44	-
Males, n (%)	6 (12.50)	7 (15.91)	0.432
Age, years	51.71 ± 10.91	55.50 ± 11.01	0.101
Height, cm	158.73 ± 8.54	158.30 ± 6.87	0.793
Weight, kg	56.68 ± 10.52	55.20 ± 9.31	0.478
BMI, kg/cm^2	22.43 ± 3.11	22.04 ± 3.50	0.576
Body fat percentage, %	29.72 ± 5.80	28.34 ± 6.95	0.302
NK cell activity, %	41.40 ± 12.87	42.55 ± 10.98	0.647
Intake rate, %	98.64 ± 2.90	98.78 ± 2.56	0.993

Values shown are mean ± standard deviation (SD). Statistical analysis was performed by Student's *t*-test for age, height, body weight, BMI, body fat percentage, and natural killer cell activity, and by the chi-square test for gender, and by Mann–Whitney U test for intake rate. n = number of subjects.

Serum biochemical data and vital signs

We found no abnormal biochemical data for liver or renal functions, blood glucose, blood lipid, or CRP that would indicate any adverse effect in the Maitake and placebo groups during the trial (Table 2). Vital signs (blood pressure, pulse, and body temperature) raised no safety concerns during the trial.

Table 2. Biochemical data.

		Week 0	Week 4	Week 8	Week 12
WBC ($\times 10^3/\mu l$)	Placebo	5.95 ± 2.39	5.74 ± 1.75	5.38 ± 1.98	5.42 ± 1.99
	Maitake	5.22 ± 1.09	5.03 ± 1.25	4.65 ± 1.16	4.80 ± 1.04
RBC ($\times 10^4/\mu l$)	Placebo	450.66 ± 34.76	454.40 ± 34.74	446.24 ± 36.43	451.24 ± 37.40
	Maitake	444.82 ± 34.25	447.45 ± 33.31	440.37 ± 32.55	446.84 ± 33.26
Hb (g/dl)	Placebo	13.62 ± 1.42	13.60 ± 1.46	13.28 ± 1.49	13.47 ± 1.61
	Maitake	13.67 ± 1.06	13.66 ± 1.09	13.36 ± 1.05	13.64 ± 1.16
Ht (%)	Placebo	41.41 ± 3.41	41.34 ± 3.72	40.67 ± 3.77	41.02 ± 4.10
	Maitake	41.46 ± 2.87	41.45 ± 2.87	40.62 ± 2.74	41.33 ± 3.11
Plt ($\times 10^4/\mu l$)	Placebo	26.44 ± 8.41	26.23 ± 7.06	25.73 ± 6.55	27.71 ± 10.21
	Maitake	23.91 ± 5.48	24.45 ± 6.11	23.20 ± 5.73	23.84 ± 5.53
AST (U/l)	Placebo	22.84 ± 7.21	22.12 ± 5.95	22.29 ± 6.59	22.82 ± 5.83
	Maitake	22.53 ± 5.77	22.45 ± 5.38	21.98 ± 5.92	22.80 ± 5.83
ALT (U/l)	Placebo	22.72 ± 14.51	22.02 ± 14.88	21.59 ± 15.52	22.14 ± 12.60
	Maitake	20.00 ± 10.83	19.49 ± 8.15	18.33 ± 8.54	20.04 ± 10.57
γ-GTP (U/l)	Placebo	26.52 ± 16.35	25.78 ± 15.64	24.73 ± 15.33	25.24 ± 13.82
	Maitake	25.65 ± 19.28	27.51 ± 23.61	24.70 ± 21.15	27.13 ± 25.19
ALP (U/l)	Placebo	199.56 ± 56.18	201.40 ± 51.68	193.63 ± 53.55	196.04 ± 54.29
	Maitake	200.00 ± 60.41	206.91 ± 65.17	193.93 ± 57.13	197.13 ± 57.04
LDH (U/l)	Placebo	199.56 ± 56.18	201.40 ± 51.68	193.63 ± 53.55	196.04 ± 54.29
	Maitake	191.51 ± 26.79	189.64 ± 25.89	186.35 ± 22.04	188.42 ± 25.90
BUN (mg/dl)	Placebo	12.64 ± 3.14	13.05 ± 2.92	13.59 ± 3.28	13.59 ± 3.71
	Maitake	12.76 ± 2.80	13.44 ± 2.51	13.51 ± 2.65	14.21 ± 3.29
CRE (mg/dl)	Placebo	0.70 ± 0.15	0.68 ± 0.12	0.69 ± 0.12	0.68 ± 0.12
	Maitake	0.71 ± 0.09	0.71 ± 0.10	0.71 ± 0.11	0.72 ± 0.12
UA (mg/dl)	Placebo	4.62 ± 1.19	4.50 ± 1.25	4.53 ± 1.14	4.49 ± 1.18

		Week 0	Week 4	Week 8	Week 12
	Maitake	4.78 ± 1.33	4.86 ± 1.38	4.89 ± 1.45	4.83 ± 1.39
FPG (mg/dl)	Placebo	88.92 ± 11.62	88.14 ± 10.35	89.37 ± 12.07	88.88 ± 11.28
	Maitake	87.96 ± 9.55	88.32 ± 9.76	89.50 ± 9.91	87.73 ± 8.62
HbA1c (%)	Placebo	5.45 ± 0.48	5.42 ± 0.45	5.47 ± 0.50	5.44 ± 0.47
	Maitake	5.38 ± 0.32	5.34 ± 0.31	5.36 ± 0.32	5.33 ± 0.30
TC (mg/dl)	Placebo	217.58 ± 35.43	229.40 ± 37.53	224.12 ± 35.32	229.20 ± 38.89
	Maitake	210.29 ± 38.16	216.94 ± 37.26	211.63 ± 34.70	222.13 ± 35.98
LDL-C (mg/dl)	Placebo	134.90 ± 32.72	140.74 ± 31.26	136.69 ± 33.34	141.92 ± 37.09
	Maitake	125.71 ± 35.91	125.57 ± 31.96	123.33 ± 30.31	131.60 ± 32.27
HDL-C (mg/dl)	Placebo	75.96 ± 21.57	76.74 ± 23.25	76.41 ± 20.27	75.78 ± 20.13
	Maitake	79.57 ± 23.21	81.00 ± 25.28	80.41 ± 22.78	81.73 ± 22.08
TG (mg/dl)	Placebo	89.02 ± 50.91	90.32 ± 45.74	89.88 ± 60.84	84.33 ± 56.30
	Maitake	83.04 ± 36.25	83.06 ± 39.08	83.41 ± 56.14	79.87 ± 46.61
CRP (mg/l)	Placebo	0.22 ± 0.71	0.08 ± 0.13	0.05 ± 0.05	0.05 ± 0.05
	Maitake	0.09 ± 0.38	0.09 ± 0.28	0.06 ± 0.08	0.03 ± 0.03

Values shown are mean ± standard division (SD). WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin, Ht, hematocrit; Plt, platelet count; AST, Aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; blood urea nitrogen, BUN; CRE, creatinine; UA, uric acid; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; CRP, c-reactive protein.

Vaccine-specific antibody responses

To establish how well influenza vaccines work during each season, influenza vaccine effectiveness is measured in observational studies. It takes 10 to 14 days following vaccination for the development of an immune response and protection. Vaccine effectiveness is an estimate of the likelihood that a vaccine will prevent influenza infection when used in clinical practice.

In general, a vaccine effectiveness of 40–60% has been estimated for the three different influenza viruses, i.e., the type A (H1N1 and H3N2) and type BX-51B strains, except in the last two seasons where only a limited effectiveness was observed against influenza induced by circulating viruses due to mismatch [4]. Immunogenicity criteria in response to vaccination are defined with respect to ages. For adults 18–60 years of age, the seroconversion rate should be >40%, with seroconversion defined as negative pre-vaccination serum conversion to an HI titer

>1:40, or a significant increase in HI antibody titer, i.e., at least a four-fold titer increase. On the other hand, the seroprotection rate, defined as the proportion of vaccinated individuals achieving a HI titer >1:40, should be >70%.

First, we analyzed the results for the total subject group followed by the subgroup analysis by age, i.e., ≤ or > 60 years of age. Figure 2 shows the seroconversion rates before (week 0) and after vaccination (weeks 8 and 12) for the total subjects. Seroconversion rates of type A/H1N1 at week 8 were 48% and 39% for the Maitake group and placebo (Fig. 2a), respectively; the difference was not statistically significant. At week 12, both rates were about 40%. As for type A/H3N2, there was no sign of increase of conversion rates in weeks 8 and 12 (Fig. 2b). With respect to type BX-51B, seroconversion rates were significantly higher for the Maitake group (38%) than the placebo group (18%) at week 12 (Fig. 2c).

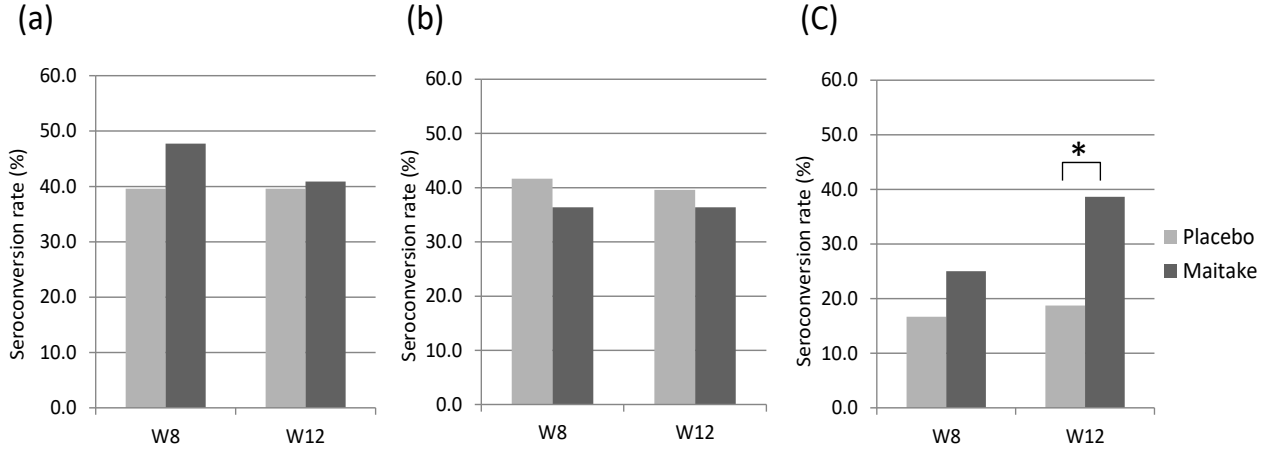

Figure 2. Change in the seroconversion rate of total subjects to influenza vaccine during the study. The analysis was carried out by the chi-square test. (a) type A/H1N1, (b) type A/H3N2, (c) type BX-51-B.

In the subclass analyses, we first assessed the seroconversion rate with respect to age. For those less than 60 years of age, the change in the seroconversion rate was not significant for any of the three virus types (Figs. 3a, 3b, 3c), among which the rate for type BX-51B tended to be higher in the Maitake group at weeks 8 and 12, but not significantly (Fig. 3c). For those more than 60 years of age, the conversion rate for type A/H1N1 was higher in the Maitake group than the placebo group at both weeks 8 and 12, with the rate in the Maitake group at week 8 (45%) being significantly higher than that of the placebo group (12%) (Fig. 4a). In a similar manner, the conversion rates for type BX-51B tended to be higher in the Maitake group, but not significantly (Fig. 4c). As for type A/H3N2, the seroconversion rates were lower in the Maitake group (Fig 4b).

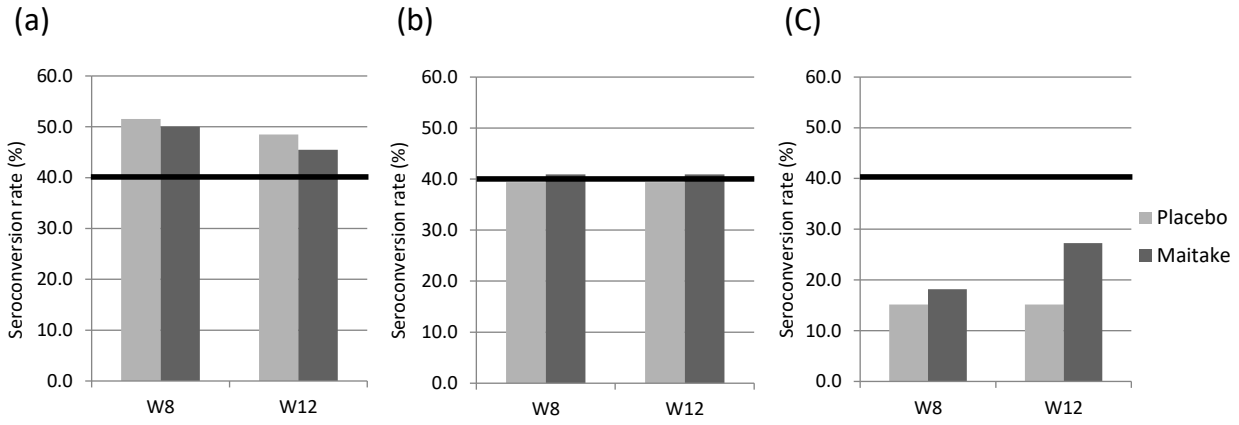

Figure 3. Change in the seroconversion rate of subjects under 60 years of age to influenza vaccine during the study. The analysis was carried out by the chi-square test. (a) type A/H1N1, (b) type A/H3N2, (c) type BX-51-B.

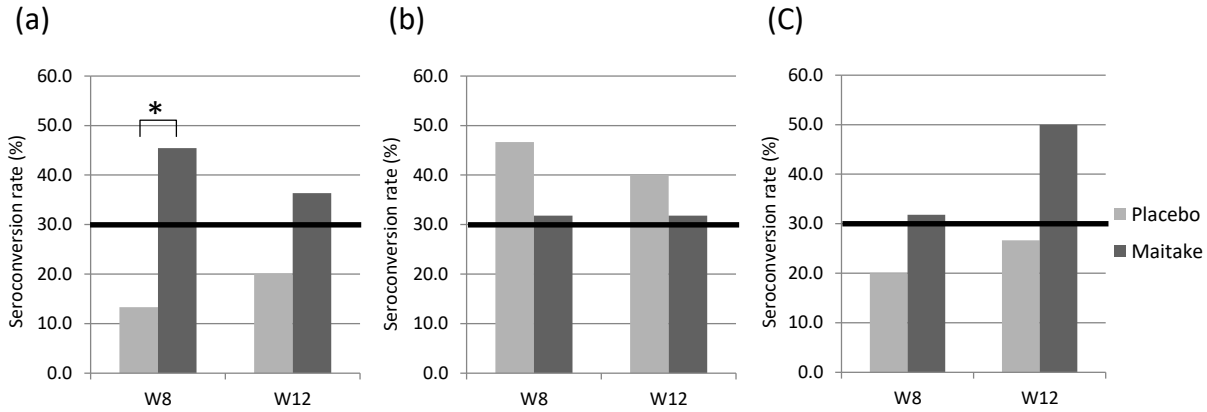

Figure 4. Change in the seroconversion rate of subjects over 60 years of age to influenza vaccine during the study. The analysis was carried out by the chi-square test. (a) type A/H1N1, (b) type A/H3N2, (c) type BX-51-B.

Secondly, we assessed the seroprotection rate with subclass analysis by age. In those under 60 years of age, we found no significant difference or tendency between the Maitake and placebo groups (data not shown). In those more than 60 years old, on the other hand, the seroprotection rate for H1N1 was positive in more than 60% of the Maitake group at weeks 8 and 12 (Fig. 5a). This result shows that when Maitake is used as an adjunct, the vaccination is effective in the older population, according to our criteria [29]. As for BX-51B, the rate in the Maitake group became significantly higher (more than 60%) than it was at baseline at week 12 (Fig. 5c), which shows its effectiveness in a similar manner. It is of note that, for the placebo group, the seroconversion rate against H3N2 was initially positive before vaccination (Fig. 5b). Although the previous season's

influenza virus was H1N1, not H3N2, according to the National Institute of Infectious Diseases in Japan, it is considered that H3N2 may have been a strain with subclinical or latent infection in the last and current seasons.

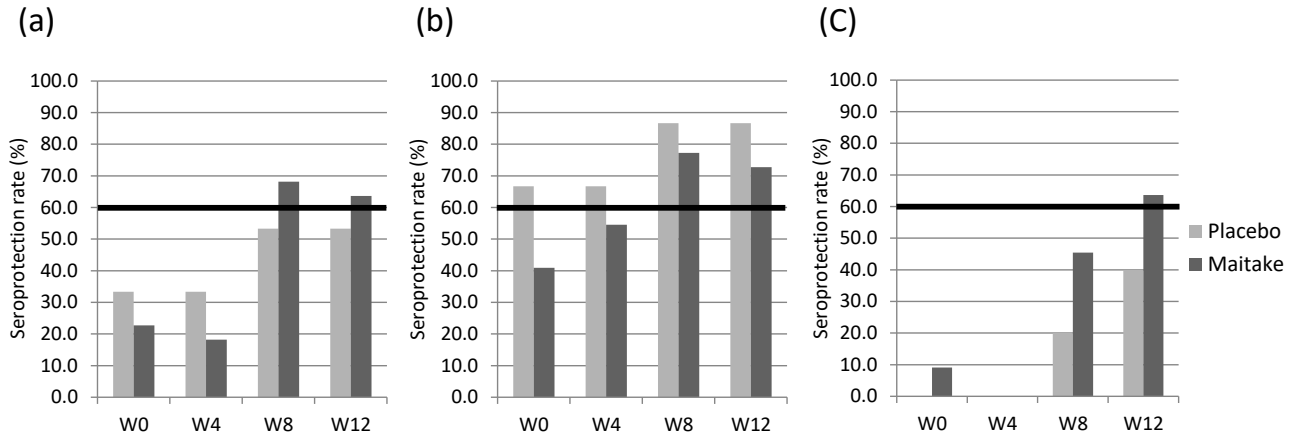

Figure 5. Change in the seroprotection rate of subjects over 60 years of age to influenza vaccine during the study. The analysis was carried out by the chi-square test. (a) type A/H1N1, (b) type A/H3N2, (c) type BX-51-B.

As for other immunological biomarkers, we measured immunological biomarkers, including NK cell activity and lymphocyte population analysis. However, none of these parameters showed any significant difference between the Maitake and placebo groups (data not shown).

Effect of Maitake on common cold symptoms

All subjects were asked to make a daily report of any common cold symptoms (headache, muscle pain and arthralgia, general malaise, fever, chill, cough, phlegm, sore throat, runny nose, stuffy nose, loss of appetite, vomiting, sneezing, and diarrhea) during the study. When it was suspected that subjects may be infected with influenza viruses, they were immediately checked by a rapid influenza immunoassay kit and examined by physicians. There was a single case of influenza infection in this study; the patient was a member of the placebo group.

Among common cold symptoms, we found that headache, muscle pain and arthralgia, general malaise, fever, chill, cough, phlegm, sore throat, runny nose, stuffy nose, loss of appetite, and vomiting were significantly improved in the Maitake group (Figs. 6a-l); sneezing and diarrhea showed no difference (data not shown). In particular, severe headache (Fig. 6a) and runny nose (Fig. 6i) symptoms were alleviated by Maitake. In brief, the occurrence rate of headache was 0.7%

for the placebo and 0.2% for Maitake ($p<0.01$), whereas the occurrence rate of runny nose was 2.2% for the placebo and 0.6% for Maitake ($p<0.01$). Maitake-associated improvements of severity were also seen for general malaise (Fig. 6c) and gastrointestinal symptoms, loss of appetite (Fig. 6k) and vomiting (Fig. 6l). On the other hand, with respect to respiratory symptoms, phlegm and cough were unchanged and worsened respectively (Figs. 6g and 6f).

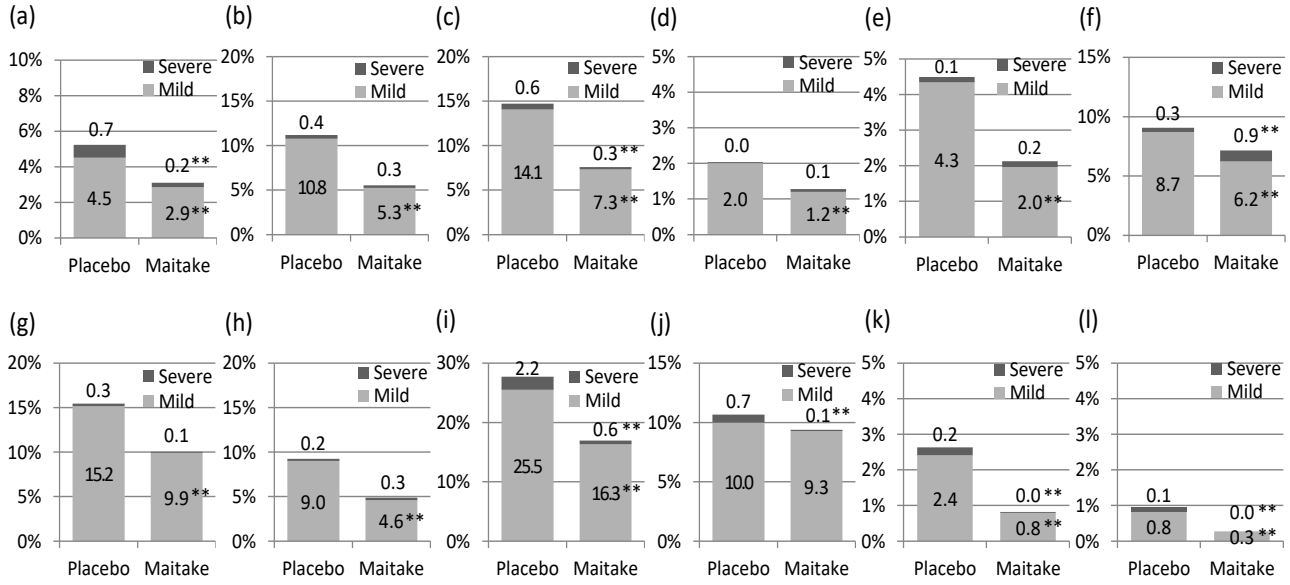

Figure 6. Frequency of severe and mild cold symptoms. Severe and mild symptoms are expressed by black and gray bars, respectively. (a) headache, (b) muscle pain and arthralgia, (c) general malaise, (d) fever, (e) chill, (f) cough, (g) phlegm, (h) sore throat, (i) runny nose, (j) stuffy nose, (k) loss of appetite, and (l) vomiting. Between-group comparisons of actual values were assessed by the chi-square test.

DISCUSSION

A comprehensive study on influenza vaccination described that antiviral medications are an important adjunct to vaccination and are effective when administered as treatment and after exposure to influenza virus [4]. An update of seasonal influenza vaccines is required every year, because of the constant evolution of circulating influenza viruses. The effectiveness of each seasonal influenza vaccine depends on the match between the selected vaccine viruses and those in circulation that year. To compensate for the mismatch, effective alternative methods to boost immunity are needed.

The annual influenza vaccination is the most effective method for preventing influenza virus

infection and its complications. Influenza vaccine is recommended for all persons aged more than 6 months who do not have contraindication to vaccination [4]. Due to the economic damage and social disruption of absences from work or school, particularly those due to pandemic influenza infection, all individuals from children to older adults are strongly encouraged to receive immunization. At present, a trivalent inactivated influenza vaccine can be used for any person aged >6 months. The most severe morbidity and mortality during the typical influenza season occurs among older adults who have chronic medical conditions [32]. According to the 10-year data pooled form 18 cohorts of community-dwelling elderly members, influenza vaccination provided significant reduction in the risk of hospitalization for pneumonia or influenza. However, among the various population groups, older adults were less responsive to the vaccine and more susceptible to influenza infection in spite of the vaccination. Thus, more immunogenic influenza vaccines, particularly ones effective for older adults, are required for persons at higher risk for influenza-related complications. In this context, the current results demonstrating enhancement of immunogenicity of influenza vaccination by Maitake mushroom would provide a novel strategy for helping to prevent endemic and pandemic influenza infection.

Whether individuals become ill when they are exposed to viruses depends on a number of factors. These include previous exposure to a similar influenza virus that has induced a complete or partial protective immunity to the current circulating virus, or exposure through vaccination with an updated matching influenza vaccine strain. It is generally accepted that young children and older adults are the most affected by seasonal influenza infection each year [4]. Older adults are less likely to be infected than children and young adults. However, when they are infected, these older adults are more likely to suffer from severe disease. In this paper, we demonstrated that Maitake is more effective within older adults. Specifically, as shown in Figures 3 and 4, both seroconversion and seroprotection of those older than 60 years were increased by Maitake intake. Improved immunity by Maitake intake would have a great impact, particularly in helping to prevent influenza infection in older adults.

There were differences in effectiveness depending on the viruses: influenza vaccination was the most effective for type H1N1 followed by Type B based on the data on seroconversion. The reason for this difference is still not understood, but it may have been due to the immunogenicity being higher for type A/H1N1, but lower for type A/H3N1. Additionally, a mismatch between the influenza vaccine and the seasonal influenza species may have been the cause of the difference in

effectiveness.

Studies on the use of medicinal mushrooms for cancer prevention and immunogenicity have been extensively performed [13-19], with a particular focus on NK cell activity. Activation of NK cells by Maitake has been well examined in relation to tumor growth, with the D-fraction markedly suppressing tumor growth, in conjunction with increases in tumor necrosis factor (TNF)-α and interferon (IFN)-γ released from spleen cells and significant increase in TNF−α expressed in NK cells [33]. Moreover, the D-fraction increased macrophage-derived IL-12, which serves to activate NK cells. These results clearly indicate that the D-fraction activates NK cells. In a similar manner, β-glucans contained in mushrooms have been shown to have a number of benefits in animal studies, and there is considerable interest in their immune-boosting abilities in humans.

As for the effect of Maitake mushrooms on immune function, an animal study using microarrays focused on genes in the liver involved in activation of immunogenicity through immunoglobulin-mediated immune responses [27]. Compared with a group of animals fed another species of mushrooms, those fed Maitake mushrooms showed a boost in the expression levels of many genes involved in the response to virus. Furthermore, an adaptive immune response was clearly seen. In this context, it seems reasonable to consider that Maitake mushrooms could modulate the immune system.

As for the mechanism of immune function, type I interferon (IFN) and NK cell activity are a representative innate cytokine and innate lymphoid cell type that play roles in innate immunity against virus-infected cells [34]. In this study, murine macrophage cells were stimulated for 10 hours with the extracts from the fruit bodies of Maitake. The extract (conditioned medium) reduced virus yields with TNF-α mRNA, leading to production of TNF-α.

In the current study, we were unable to provide any solid evidence of the enhancement of either IFN-γ production or NK cell activity. However, we speculate that the sample number of the current study was insufficient, and/or the NK cell activities of subjects were relatively higher, which hindered detection of the effectiveness of Maitake on these biomarkers. Nevertheless, we considered that Maitake would be useful to stimulate not only NK cell activity, but also IFN-γ production. To prove this, it would be worthwhile to perform these experiments in more detail with an increase of the sample number and/or selection of samples with low NK cell activity. We should also investigate the role of regulatory T cells and other potential factors to clarify the detailed mechanism of the immunomodulatory effect of Maitake mushrooms.

In spite of our failure to identify any evidence of IFN-NK cell action, we did demonstrate that Maitake mushroom played a role in the stimulation of immune mechanisms. In a previous study using DNA microarray analysis to profile the hepatic gene expression of mice fed three different mushroom species, the expression of other interferon-regulated virus resistance genes was shown to increase significantly in response to Maitake intake in association with increases in molecules of TLR3 and cytokines [27]. The expression level of TLR3, which acts on signal transduction of innate immunity, was upregulated compared with that in the control group. TRL3 is a particularly important receptor in the signal transduction pathway for recognition of influenza virus. Maitake contains a unique form of β-glucan referred to as β -1,3;1,6-glucan with xylose and mannose. β-Glucans are ubiquitously found in both bacterial and fungal cell walls, and act on several immune receptors, including several types TLRs [12]. Taking all these findings into consideration, it is hypothesized that Maitake dietary intake enhanced the protection against infection by influenza and other viruses by an increase in interferon production, mainly via TLR3, the induction of virus resistance genes, and the activation of NK cells.

In addition to their effectiveness against influenza infection, Maitake mushrooms are effective for suppressing various stresses, such as dyslipidemia and hyperglycemia, possibly due to the effect of their high levels of β-glucans [35]. Another report showed that Maitake mushrooms are also rich in minerals, amino acids and vitamins, which are essential for a healthy body in warding off intrusive microorganisms. With respect to nutrients, it is of interest that the effects of the log and sawdust substrate compositions were different [36], suggesting that the functionality of Maitake would vary according to the method of cultivation. It is speculated that the abilities of Maitake to fight the common cold and boost antibody production against influenza may both be attributable to specific nutrients contained in Maitake.

In Japan, Maitake mushrooms are used in hot pot dishes in winter to prevent infectious diseases in general, not just for influenza infection. In this study, we demonstrated that Maitake intake significantly and widely suppressed the emergence of a variety of clinical symptoms related with the common cold, including cough, fever, and general malaise. It should be noted that severe symptoms were effectively ameliorated by Maitake mushroom, clearly indicating that Maitake mushrooms exerted their effects against virus infection by augmenting immunogenicity.

In view of public health, all subjects received a vaccine against influenza virus in the study; we should take this into consideration when evaluating the immune-boosting function of Maitake

mushrooms shown here. Morevoer, it seems reasonable to expect that Maitake will also potentiate the host immune response without vaccination. Therefore, we need further clinical trials to evaluate the efficacy of Maitake mushrooms on host immune responses without vaccination.

Lastly, we demonstrated that continuous intake of Maitake mushrooms improved influenza titers on influenza type A virus H1N1 and type BX-51B virus, particularly for older adults, in this study. Maitake mushrooms are a useful adjunct for seasonal influenza vaccines, and are usually recommended for vulnerable populations that also are poorer immune responders due to aging or chronic diseases. Several attempts to improve the vaccine have been explored over the last 10–15 years, including an experimental live, attenuated, intranasally administered trivalent influenza vaccine [1]. New antiviral drugs based on the structure of the neuraminidase molecule were assessed and found to be effective [2]. We demonstrated here that Maitake mushrooms suppressed clinical symptoms effectively. Further, the current study provided good evidence that Maitake mushrooms activate both adaptive and innate immune responses. Based on these findings, we expect that Maitake mushrooms potentiate host defense systems and protect humans from not only influenza virus, but also other pathogenic viruses and bacteria. It is reported that a fermented papaya preparation supplementation for 1 month resulted in higher concentration of salivary IgA and lysozyme together with antioxidant enzymes in the human airway [37]. In this context, the combined supplementation of this fermented nutraceutical with Maitake mushroom would be very effective in preventing and mitigating acute respiratory illness such as seasonal influenza.

List of Abbreviations: HI, hemagglutination inhibition; IFN, interferon; NK, natural killer; TLR, toll-like receptor.

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