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Maitake Mushroom (*Grifola frondosa*) Enhances Cognitive Function in Healthy Older Japanese: A Randomized, Double-Blind, Placebo-Controlled Trial

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Summary Extended longevity has resulted in an increased number of patients with dementia. Therefore, new methods for treating and preventing dementia are highly desirable. Multiple health benefits have been reported for *Grifola frondosa* (maitake mushrooms). In this study, we investigated the safety and effects of continuous intake of maitake mushrooms on cognitive function in healthy older individuals. We conducted a randomized, double-blind, placebo-controlled trial over 18 wk targeting 47 healthy Japanese individuals aged 60 y or older. The individuals were randomly divided into three groups: a Y10M group consumed bread containing 50 g of the maitake mushroom strain Y10M, a C5304 group consumed bread containing 50 g of the maitake mushroom strain C5304, and a placebo group consumed bread without maitake mushrooms. No participants discontinued the intervention due to adverse effects in any group. The Y10M group showed a significant improvement in cognitive assessment based on the Japanese version of the Montreal Cognitive Assessment (MoCA-J) score compared to the placebo group after the intervention. However, no difference was observed in the MoCA-J scores between the C5304 and placebo groups. The activity of natural killer cells, a type of immune cell, was also significantly augmented in the Y10M group and positively correlated with MoCA-J scores. These results suggest that improved cognitive function is, at least in part, attributable to the activation of the immune system. This study suggests that consumption of maitake mushrooms may help inhibit the decline in cognitive function in healthy older individuals.

Key Words maitake mushroom, cognitive function, mild cognitive impairment, risk of dementia, NK cells, safety

In recent years, the prevalence of dementia has rapidly increased with an increase in average life expectancy. The onset of dementia decreases memory and judgment abilities, which markedly impedes social interactions and decreases the quality of life (QOL) of both patients and their caregivers. The onset of dementia is reportedly caused by the accumulation of β-amyloid (Aβ) in Alzheimer's disease (AD) (*1*), as well as by multiple related diseases including hypertension and diabetes (*2–4*). However, the pathological mechanism has not yet been fully elucidated and no curative treatment has been established. Therefore, many studies have investigated methods to prevent the onset of dementia and to control disease progression. Because recovery from mild cognitive impairment (MCI), an intermediate state between healthy cognition and dementia, is sometimes possible (*5*), preventing the progression of MCI to dementia may be a crucial strategy.

Several studies have reported a correlation between lifestyle habits and prevention or slowing of dementia progression (*6–9*). In particular, diet-based approaches are considered highly effective as they are easy to incorporate into daily life. Previous studies investigating the correlations between diet and dementia have reported that mushroom consumption lowers the risk of dementia onset (*10*) and improves cognitive functions such as perceptual speed, executive function, semantic memory, and episodic memory (*11*). Intake of the mushroom yamabushitake (*Hericium erinaceus*), particularly, improves cognitive function (*12*). However, the effects of

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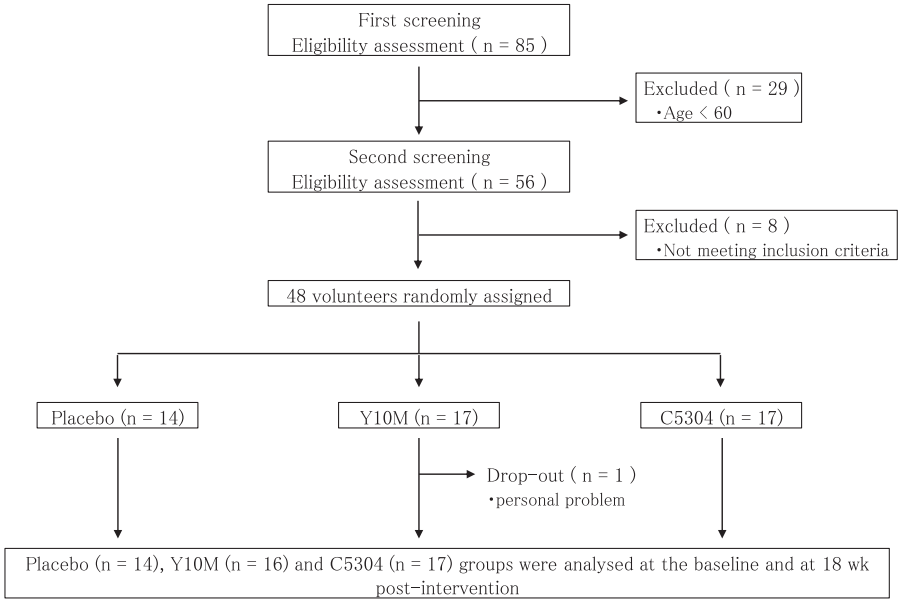


Fig. 1. Flow diagram of the study participants.

most edible mushrooms on cognitive functions remain poorly understood.

The maitake mushroom (*Grifola frondosa*), an edible mushroom commonly consumed in Asia, has been reported to have a range of health-promoting effects, such as lowering blood pressure and blood glucose levels (*13*), lowering blood lipid and cholesterol levels (*14*), improving intestinal flora (*15*), and activating the immune system against tumors (*16*, *17*) and infections (*18*, *19*). Maitake mushrooms have been reported to improve cognitive function in animal models of cognitive decline (*20*, *21*). Suggested mechanisms for maitake mushrooms improving cognitive function are protecting neurons through anti-oxidative effects (*20*), and the removal of Aβ through immune activation (*21*). The relationship between immune activation by maitake mushroom and improved cognitive function is particularly interesting because systemic immunity plays an important role in brain function (*22*); for example, natural killer (NK) cells induce Aβ clearance by improving microglial function (*23*). However, the effects of maitake mushrooms on cognitive function in healthy individuals have not been clarified. In the present study, we evaluated whether daily consumption of maitake mushrooms helped maintain cognitive function in a randomized, double-blind, placebo-controlled 18-wk trial using two strains of maitake mushrooms targeting healthy older people.

MATERIALS AND METHODS

Study design and ethical considerations. This randomized double-blind placebo-controlled trial was approved by the Ethics Committee of Kato Hospital, Jinjukai Healthcare Corporation (approval no. 2022-0022). The study was conducted under the supervision of a physician in accordance with the Ethical Guidelines for Medical and Biological Research Involving Human Subjects and the Declaration of Helsinki. All the participants were unpaid volunteers. After receiving written and verbal explanations of the purpose, methods, and possible side effects of the study, the participants voluntarily provided written informed consent to participate. This study was registered as a clinical trial in the University Hospital Medical Information Network Center (UMIN ID: UMIN000050636).

Subjects. After providing informed consent, healthy volunteers from Kawamoto-machi, Oochi-gun, Shimane Prefecture, Japan were pre-screened: those who met the inclusion criteria, but none of the exclusion criteria, were enrolled. After excluding 37 of the 85 recruited individuals who did not meet the eligibility criteria, 48 participants (19 male and 29 female) were included in the study (Fig. 1).

The inclusion criteria were:

① Age between 60 and 85 y at the time of consent
② Screening for an overall Mini-Mental State Examination (MMSE) overall score of at least 24
③ No diseases that could affect study implementation
④ Provided written consent to participate in the study themselves
⑤ Can converse in Japanese

The exclusion criteria were:

① Receiving hospital treatment and/or medication for dementia, osteoporosis, liver dysfunction, renal dysfunction, or malignant tumors
② Hospital visits and/or treatment for an immune function disorder, e.g., with steroids or immune suppressors
③ Currently pregnant
④ Fasting serum triglycerides of 199 mg/dL or higher
⑤ Regularly taking prescribed muscle enhancers, such as anabolic steroids
⑥ Regularly taking prescription digestive aids
⑦ Allergic to maitake mushroom

Sample size. The sample size was calculated based on the change in MMSE scores before and after the

intervention in the maitake and placebo groups, with reference to similar studies (*7*, *24*, *25*). Based on data from a previous randomized controlled trial involving a similar population (*7*, *24*, *25*), a mean difference of 1.4 points (SD=2.0) between groups was expected. Power calculations were performed using the Power and Sample Size Calculation Software Version 3.1.2 (Vanderbilt University, Nashville, TN, USA). Assuming 80% power and a two-sided alpha of 0.05, at least 13 participants per group were required to achieve statistical significance. Considering a dropout rate of 5%, 14 participants per group was deemed the minimum requirement.

Test foods. A previous study reported that daily intake of 6.8 g dried powder of maitake mushroom strain Gf433 (equivalent to 68 g of fresh maitake mushrooms) improved common cold symptoms (*19*). It has been suggested that maitake mushroom extract is comparable to *Lentinus edodes* (shiitake mushroom) extract in activating the immune system (*26*), and that 5 g of dried shiitake mushroom powder (equivalent to 50 g of fresh shiitake mushrooms) activates the immune system (*27*). Accordingly, we set the daily maitake mushroom dose to 50 g, which is considered a safe and effective dose. The test food was bread containing maitake mushroom strains Y10M or C5304, manufactured by YUKIGUNI FACTORY Co., Ltd. (Niigata, Japan). Fifty grams of Y10M corresponds to approximately 3.3×10^9 cells and contains 1,193.7 mg of β-glucan, and 50 g of C5304 corresponds to approximately 3.4×10^9 cells and contains 1,404.0 mg of β-glucan. The participants consumed two pieces of bread, each 15 cm long×5 cm wide and weighing 55 g, containing a daily dose of heated maitake mushroom paste. The placebo was bread without the maitake mushroom paste. Nutritional components of the placebo and test breads manufactured by Musashino Foods Co., Ltd. (Saitama, Japan) are shown in Table 1. To mask smell and taste of the maitake mushrooms, tomato and onion powders were added to each of the placebo and test breads. In a blinded sensory test involving 37 individuals the average percentage of correct identification was 51% between the placebo and test breads, indicating that the placebo and test breads could not be distinguished by appearance, smell, or taste. Participants ingested food at any time they chose.

Study methods. This was a randomized, double-blind, placebo-controlled trial. Participants were randomly assigned to the placebo, Y10M, or C5304 groups using stratified randomization based on age, sex, and baseline MMSE scores to ensure balanced allocation across groups. Both the participants and investigators involved in the outcome assessment and data analysis were blinded to the group assignments. Randomization codes were prepared by an independent researcher who was not involved in the study and were concealed until the completion of statistical analyses. The placebo group was given placebo bread, the Y10M group was given Y10M containing bread and the C5304 group was given C5304 containing bread. The participants were asked to complete daily diaries indicating whether they had consumed the assigned bread, and adherence to the study protocol was assessed based on these records. The study was conducted from December 2022 to April 2023, with an intervention period of 18 wk. Before implementation, a briefing was held in which the participants were requested not to make any major changes in their lifestyle, e.g., such as diet, exercise, or medication, during the study. On the day of the test, participants did not eat breakfast. A doctor interview was conducted; physical measurements, including blood pressure and blood collection, were performed; and cognitive function was evaluated. A consultation system was established at Kato Hospital to ensure safety during the intervention period. In addition, 12 wk after the intervention, the participants underwent a doctor's interview, which served as a health survey, and fasting blood samples were collected to monitor blood parameters.

Table 1. Nutrient composition of the placebo and maitake bread.

	Placebo	Y10M	C5304
Energy (kcal)	285.3±3.2	249.2±2.6	249.1±2.5
Protein (g)	11.3±0.2	10.6±0.2	10.5±0.1
Fat (g)	4.8±0.1	3.8±0.1	4.0±0.1
Carbohydrate (g)	50.5±0.5	45.0±0.5	45.0±0.5
Fiber (g)	2.5±0.1	3.9±0.1	4.3±0.1
Moisture (g)	31.9±0.7	39.1±0.7	39.1±0.6
Ash (g)	1.6±0.0	1.5±0.0	1.5±0.0
Sodium (mg)	441.5±5.0	364.3±1.0	372.0±3.0
Ergosterol (mg)	1.8±0.0	24.1±0.1	27.2±0.6
β-glucan (mg)	107.5±2.8	871.0±26.0	1,002.2±14.8

Nutritional values represent the nutritional content per 100 g of placebo, Y10M, or C5304 bread. β-glucans were analyzed by YUKIGUNI FACTORY Co., Ltd. (Niigata, Japan), $n=3$. Other nutritional components were analyzed by Japan Food Research Laboratories (Tokyo, Japan), $n=4$. Data are presented as mean±standard error (SE).

Physical measurements, body composition, and diet evaluation. Body weight, height, waist circumference, and diastolic and systolic blood pressure were measured by a nurse. Body composition was assessed by bioelectric impedance analysis using WB-150 (Tanita Corp., Tokyo, Japan). To investigate the effects of the intervention on the health status and lifestyle of each study group, we surveyed their lifestyles, including medical history. The amount of food consumed before and after the intervention was evaluated by using an approved brief self-administered diet history questionnaire (BDHQ) (*28*). The BDHQ consists of four pages that list the foods commonly consumed in Japan, mainly those listed in The National Health and Nutrition Survey. Participants were required to select the frequency of consumption of 58 food/beverage portions to estimate the amount ingested over the previous month, and the content of 98 nutritional factors was inferred using a specialized

Table 2. Participant characteristics.

	Baseline			*p*-value
	Placebo	Y10M	C5304	
Anthropometry				
Sex (male/female)	14 (6/8)	16 (6/10)	17 (6/11)	0.914
Age (y)	70.7±1.8	70.0±1.4	70.1±1.5	0.946
Height (cm)	160.4±3.3	156.6±2.6	157.1±1.9	0.557
Body weight (kg)	58.3±2.7	56.0±3.7	59.0±2.4	0.763
Body mass index (kg m^{-2})	22.4±0.7	22.5±0.9	23.8±0.8	0.429
Waist circumference (cm)	86.0±1.7	84.6±2.8	89.0±2.6	0.432
Education (y)				
≦12 y (%)	7 (50.0%)	6 (37.5%)	9 (52.9%)	
>12 y (%)	7 (50.0%)	10 (62.5%)	8 (47.1%)	

Data are presented as the mean±SE. Statistical analyses were performed using analysis of variance. C5304, the C5304 bread-fed group, $n=17$; Placebo, bread without Y10M and C5304, $n=14$; Y10M, the Y10M bread-fed group, $n=16$.

computer algorithm (*28*).

Blood analysis. After confirming that the participants had not eaten breakfast, blood samples were collected in the morning before the intervention commenced and at 12 and 18 wk post-intervention. Blood was separated into red blood cells, peripheral blood mononuclear cells (PBMCs), serum, and plasma by centrifugation. Biochemical markers, including hemoglobin A1c (HbA1c) and ergosterol, were measured in the serum. Glucose levels and NK cell activation were evaluated in the plasma and PBMC fractions, respectively. The analyses of biochemical markers, HbA1c, and blood glucose were outsourced to SRL, Inc. (Tokyo, Japan). The remaining serum and plasma were stored at −80°C.

β-Glucan measurement. β-Glucan concentration quantified using K-YBGL kits (Megazyme, Ireland). β-Glucan was determined by the following method. Freeze-dried bread powder was boiled in 5 mL of 50% ethanol (v/v) for 5 min. After centrifugation (1,700 ×*g*, 10 min), the precipitate was washed with 5 mL of 50% ethanol. The α-glucan, trehalose and sucrose were decomposed according to the kit instructions and then removed from the sample by rinsing twice with 10 mL of Milli-Q water at 50°C. The resulting pellet was used for β-glucan quantification according to the kit instructions.

Ergosterol measurement. Ergosterol levels were measured as previously described (*29*). Acetonitrile and 25-hydroxy vitamin D2-d6 (internal standard) were added to the serum. After centrifugation, the supernatant was extracted with ethyl acetate, and derivatization was performed using Amplifex® Diene reagent (SCIEX, MA, USA). The derivatized compounds and internal standards were analyzed using liquid chromatography-tandem mass spectrometry on a Shimadzu 8045 triple quadrupole instrument (Shimadzu Corp., Kyoto, Japan).

NK cell activation. Blood samples were collected in mononuclear cell separation tubes and separated into plasma and PBMCs. PBMCs were incubated overnight at 4°C with a monoclonal antibody mix containing anti-CD3, anti-CD56 and anti-CD69 antibodies (all from BIOLEGEND). The labeled cells were analyzed by fluorescence-activated cell sorting using a FACSAria III flow cytometer (BD Biosciences, San Jose, CA, USA). NK cells were characterized by a CD3 negative and CD56 positive phenotype.

Evaluation of cognitive function. To overcome the expected difficulties in determining the effects of this intervention, we used three different tests to evaluate cognitive function. In addition to the MMSE, which is widely used worldwide as a dementia diagnostic test (*30*), we implemented the Revised Hasegawa's Dementia Scale (HDS-R), which is commonly used in Japan (*31*), and the Japanese version of the Montreal Cognitive Assessment (MoCA-J), which has been shown to be effective for MCI identification, to detect small changes in cognitive function (*32*, *33*).

Statistical analysis. The per protocol set and safety analysis set were applied to analyze efficacy and safety outcomes, respectively. Data distribution was analyzed using the Shapiro–Wilk test. The baseline values of subject characteristics were analyzed using analysis of variance, with "group" as the between-participant variable. Post-intervention values of blood pressure, biochemistry, BDHQ, and ergosterol were analyzed using analysis of covariance (ANCOVA) adjusted for baseline values, age, and sex. The changes in ergosterol levels were adjusted for baseline age and sex. Post-intervention values of the MMSE, HDS-R, MoCA-J, and NK cell activity were analyzed using ANCOVA adjusted for baseline values, age, sex, and education (y). Changes in MMSE, HDS-R, MoCA-J scores, and NK cell activity were analyzed using analysis of covariance adjusted for age, sex, and education (y). Post-hoc analyses were performed using the Bonferroni test following ANCOVA. Pairwise comparisons were conducted among the placebo, Y10M, and C5304 groups to identify significant differences. Correlations between the MoCA-J scores

Table 3. Physical and hematological parameters at baseline and post-intervention.

		Placebo	Y10M	C5304	*p*-value
Blood pressure (BP)					
Systolic BP (mm Hg)	Baseline	148.1±6.4	153.7±3.7	151.9±5.2	
	Post-intervention	152.4±5.2	149.8±3.8	144.8±4.0	0.151
Diastolic BP (mm Hg)	Baseline	83.5±2.4	88.3±3.0	84.5±2.6	
	Post-intervention	81.9±2.1	85.9±2.3	82.1±2.7	0.794
Blood biochemistry					
AST (U L^{-1})	Baseline	20.8±0.8	24.5±1.6	22.6±1.4	
	Post-intervention	21.2±0.8	23.1±1.1	22.5±1.4	0.928
ALT (U L^{-1})	Baseline	18.6±1.5	17.5±1.4	20.0±1.5	
	Post-intervention	17.8±1.2	18.1±1.7	20.8±1.9	0.340
γ-GTP (IU L^{-1})	Baseline	24.6±3.3	29.5±4.1	21.0±1.5	
	Post-intervention	21.8±3.2	29.5±4.3	20.3±1.6	0.347
Albumin (g dL)	Baseline	44.5±0.4	44.3±0.6	45.1±0.8	
	Post-intervention	44.4±0.6	45.8±0.7	44.6±0.7	0.064
Total cholesterol (mg dL^{-1})	Baseline	206.8±7.5	211.1±6.3	205.6±6.4	
	Post-intervention	209.9±8.6	233.3±7.0	211.9±7.0	0.102
Triglyceride (mg dL^{-1})	Baseline	94.5±11.1	79.2±12.3	87.5±5.7	
	Post-intervention	90.5±9.6	97.5±14.5	92.8±9.3	0.815
Creatinine (mg dL)	Baseline	6.9±0.3	7.2±0.5	7.1±0.3	
	Post-intervention	6.8±0.3	6.9±0.3	7.0±0.3	0.553
Blood glucose (mg dL^{-1})	Baseline	106.3±3.8	102.4±3.6	104.4±3.4	
	Post-intervention	93.5±6.2	97.7±2.4	97.4±2.9	0.278
HDL-C (mg dL^{-1})	Baseline	67.0±4.9	75.4±7.1	66.9±3.4	
	Post-intervention	68.9±4.2	80.4±6.8	68.8±3.7	0.168
LDL-C (mg dL^{-1})	Baseline	118.2±6.4	110.8±5.0	117.1±4.6	
	Post-intervention	117.2±8.0	122.9±5.1	124.1±5.6	0.218
HbA1c (NGSP) (%)	Baseline	6.0±0.1	5.9±0.1	6.0±0.1	
	Post-intervention	5.9±0.1	5.7±0.1	5.9±0.1	0.466
Hematological parameters					
WBC ($\times 10^2\ \mu L^{-1}$)	Baseline	50.8±3.4	55.9±4.2	52.0±2.5	
	Post-intervention	53.0±4.1	57.1±5.0	51.9±2.5	0.854
RBC ($\times 10^4\ \mu L^{-1}$)	Baseline	468.4±12.7	446.8±11.9	446.9±10.4	
	Post-intervention	457.2±12.2	446.0±10.6	441.9±11.3	0.664
Hemoglobin (g dL^{-1})	Baseline	14.3±0.3	14.0±0.4	13.7±0.3	
	Post-intervention	14.1±0.3	13.8±0.3	13.8±0.4	0.852
Hematocrit (%)	Baseline	44.2±1.0	42.6±1.1	42.1±0.9	
	Post-intervention	43.1±0.9	42.0±0.8	41.7±1.0	0.977
MCV (fL)	Baseline	94.6±1.0	95.6±1.3	94.4±1.0	
	Post-intervention	94.5±0.9	94.5±1.3	94.6±1.0	0.068
MCH (pg)	Baseline	30.7±0.4	31.3±0.4	30.7±0.4	
	Post-intervention	30.9±0.4	31.0±0.5*	31.2±0.4	0.034
MCHC (g dL^{-1})	Baseline	32.4±0.2	32.7±0.2	35.5±0.2	
	Post-intervention	32.7±0.3	32.8±0.2	32.9±0.2	0.525
Platelet ($\times 10^4\ \mu L^{-1}$)	Baseline	21.4±1.5	28.1±1.7	24.8±1.5	
	Post-intervention	21.1±1.2	28.1±1.4	24.0±1.6	0.267

Data are presented as the mean±SE. All reported *p*-values were derived from ANCOVA models adjusted for baseline values, age, and sex, with Bonferroni correction applied to pairwise comparisons. Significant differences were observed compared with the placebo group using the post-hoc test, $0.01 < {}^{*}p < 0.05$. ALT, alanine transaminase; AST, aspartate transaminase; C5304, the C5304 bread-fed group, $n=17$; γ-GTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NGSP, national glycohemoglobin standardization program; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; RBC, red blood cell; WBC, white blood cell; Y10M, the Y10M bread-fed group, $n=16$.

Table 4. Dietary intake evaluated using the BDHQ at baseline and post-intervention.

		Placebo	Y10M	C5304	*p*-value
Energy (kcal)	Baseline	1,987.4±129.6	2,065.6±202.0	2,040.2±195.4	
	Post-intervention	1,841.6±133.2	1,781.4±155.5	1,950.2±163.3	0.352
Protein (g/d)	Baseline	86.6±6.6	89.8±11.9	78.8±8.3	
	Post-intervention	76.9±5.5	72.9±7.8	76.7±5.9	0.278
Fat (g/d)	Baseline	64.0±6.1	61.7±6.9	59.1±6.4	
	Post-intervention	60.7±4.9	55.0±4.7	60.6±6.2	0.427
Carbohydrate (g/d)	Baseline	246.1±15.3	258.2±29.5	271.7±24.3	
	Post-intervention	224.8±16.1	218.5±22.4	249.4±22.0	0.443
Total dietary fiber (g/d)	Baseline	15.1±1.2	16.8±2.3	13.8±2.1	
	Post-intervention	13.9±1.0	12.5±1.4	12.9±1.4	0.037

Data are presented as the mean±SE. All reported *p*-values were derived from ANCOVA models adjusted for baseline values, age, and sex, with Bonferroni correction applied to pairwise comparisons. BDHQ, Brief self-administered diet history questionnaire; C5304, the C5304 bread-fed group, $n=17$; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=16$.

Table 5. Serum ergosterol concentration at baseline and post-intervention.

		Placebo	Y10M	C5304	*p*-value
Ergosterol (ng/mL)	Baseline	1.0±0.2	0.6±0.1	1.0±0.3	
	Post-intervention	0.4±0.1	2.8±0.5***	3.1±0.5***	<0.001

Data are presented as the mean±SE. All reported *p*-values were derived from ANCOVA models adjusted for baseline values, age, and sex, with Bonferroni correction applied to pairwise comparisons. Significant differences were observed compared with the placebo group by post-hoc test; ***$p<0.001$. C5304, the C5304 bread-fed group, $n=16$; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=16$. In the C5304 group, one sample was excluded from ergosterol measurement due to insufficient serum volume, resulting in a final sample size of $n=16$.

and NK cell activity were evaluated using partial correlation coefficients adjusted for age, sex, and education (y). The difference in mean fluorescence intensity (ΔMFI) between the baseline and post-intervention samples was calculated as $\Delta MFI=((MFI_{baseline}-MFI_{post\text{-}intervention})/MFI_{baseline})\times100\%$. Safety and cognitive function were assessed as the primary and secondary outcomes, respectively. All analyses were performed using SPSS (version 23.0; IBM, Tokyo, Japan), with statistical significance set at $p<0.05$.

RESULTS

Clinical features and dietary intake

Overall, 47 participants completed the 18-wk trial (Fig. 1). One male participant was unable to complete the trial owing to hospitalization for foot surgery. No participants discontinued the intervention due to adverse effects such as abdominal pain, palpitations, or allergic reactions in the Y10M and C5304 groups. Table 2 shows the participants' background information. There were no significant differences in these parameters between any two groups. No significant differences were observed in these parameters between any two groups after intervention (data not shown). The final participants showed high adherence to the study protocol for 18 wk (98.4±0.02% for the placebo group, 95.9±0.03% for the Y10M group, and 96.6±0.03% for the C5304 group), although this could not be confirmed for some data for personal reasons.

Blood pressure and blood biochemistry

Post-intervention, the red cell mean corpuscular volumes were significantly different between the Y10M and C5304 groups; however, the values were within the normal range and were not considered clinically problematic (Table 3). No differences were observed in any of the other parameters between the groups. Although the triglyceride level in the Y10M group appeared to increase after intervention relative to baseline, the difference was not significant ($p=0.170$) (Table 3). A significant difference was observed in the mean corpuscular hemoglobin concentration (MCHC) level between post-intervention and baseline in the C5304 group ($p<0.001$) (Table 3); however, the post-intervention MCHC value were within the normal range. Since the 18-wk intervention did not increase or decrease any physical or hematological parameters beyond the standard values, it appeared that no adverse events related to lipid metabolism, liver function, kidney function, or the hematopoietic system occurred.

Nutritional intake

The mean nutrient intake based on the BDHQ was significantly different in the total dietary fiber post-intervention using ANCOVA; however, there was no significant difference between any pair of groups (Table 4).

Since there were no significant between-group differences, it is thought that there was no difference in nutritional status between the groups. Furthermore, there were no differences in any of the other parameters between the groups (Table 4), indicating that the consumption of bread containing 50 g of maitake mushrooms did not influence the nutritional status during the intervention period.

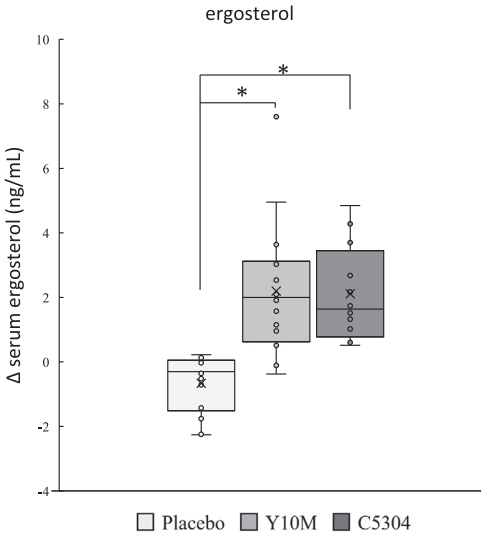


Fig. 2. Changes in serum ergosterol concentration from baseline to post-intervention. The data are presented as box-and-whisker plots. The diagram shows the median, and the box boundaries show the first and third quartiles. Statistical analyses were performed using ANCOVA adjusted for age and sex. Significant differences were observed compared with the placebo group by post-hoc test, $0.01<^{*}p<0.05$. C5304, the C5304 bread-fed group, $n=16$; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=16$. In the C5304 group, one sample was excluded from ergosterol measurement due to insufficient serum volume, resulting in a final sample size of $n=16$.

Blood ergosterol

Ergosterol is a lipid found almost exclusively in fungi, including maitake mushroom (*34*). Additionally, blood ergosterol has been reported as a biomarker of maitake mushroom intake (*29*). In the present study, we measured blood ergosterol concentrations to confirm maitake mushroom consumption and absorption in the Y10M and C5304 groups. Blood ergosterol concentrations in the Y10M group were obtained from a recently published study (*29*). The post-intervention blood ergosterol concentrations were significantly higher in the Y10M ($p<0.001$) and C5304 ($p<0.001$) groups than in the placebo group (Table 5). These changes were significantly greater in the Y10M ($p<0.001$) and C5304 ($p<0.001$) groups than in the placebo group (Fig. 2).

Cognitive function

The mean HDS-R, MMSE, and MoCA-J total scores of the 30 participants before intervention were (28.7±0.3)/30, (29.3±0.2)/30, and (26.5±0.5)/30, respectively (Table 6). Regarding post-intervention scores, no significant differences were observed in any of the groups in the MMSE and HDS-R total scores, whereas a significant difference was observed between the Y10M and placebo groups ($p=0.045$) in the MoCA-J total score (Table 6). In change score, no significant differences were observed between the groups in the MMSE or HDS-R total scores, whereas a significant difference was found between the Y10M and placebo groups ($p=0.040$) in the MoCA-J total score (Fig. 3A). Analysis of the MoCA-J subscales revealed, the Y10M group showed a significantly higher change score in the sub-item "memory" score ($p=0.024$) than the placebo group (Fig. 3B).

NK cell activation

To investigate whether the immune system contributed to improved cognitive function scores, we used flow cytometry to quantify NK cell activation, specifically analyzing peripheral blood NK cells, before and after 18 wk of intervention. NK cells are characterized by

Table 6. Cognitive scores at baseline and post-intervention.

Cognitive index (score)		Placebo	Y10M	C5304	*p*-value
MMSE	Baseline	29.1±0.4	29.5±0.2	28.8±0.3	
	Post-intervention	28.6±0.3	29.4±0.3	29.0±0.3	0.297
HDS-R	Baseline	28.3±0.6	29.1±0.3	28.4±0.5	
	Post-intervention	28.6±0.4	29.3±0.3	29.1±0.3	0.555
MoCA-J	Baseline	26.9±2.7	26.2±2.7	24.8±2.8	
	Post-intervention	25.9±3.0	27.3±1.7*	25.4±2.1	0.045
MoCA-J subscale "memory"	Baseline	3.7±0.4	2.8±0.5	2.3±0.5	
	Post-intervention	2.8±0.4	3.8±0.4	2.8±0.4	0.083

Data are presented as the mean±SE. All reported *p*-values were derived from ANCOVA models adjusted for baseline values, age, sex, and years of education, with Bonferroni correction applied to pairwise comparisons. Significant differences were observed compared with the placebo group using the post-hoc test, $0.01<^{*}p<0.05$. C5304, the C5304 bread-fed group, $n=17$; HDS-R, Hasegawa's dementia scale revised; MMSE, Mini-Mental State Examination; MoCA-J, Japanese version of the Montreal Cognitive Assessment; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=16$.

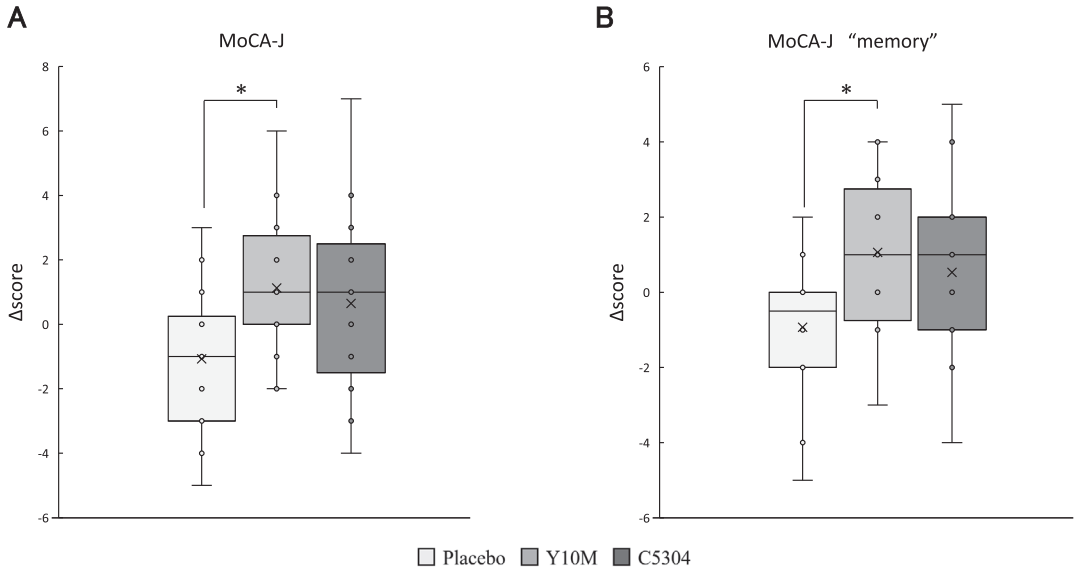


Fig. 3. Changes in MoCA-J score from baseline to post-intervention. Change in (A) MoCA-J total score and (B) MoCA-J subscale "memory" score from baseline to after 18 wk of intervention. The data are presented as box-and-whisker plots. The diagram and box boundaries are the same as Fig. 2. Statistical analyses were performed using ANCOVA adjusted for age, sex, and education (y). Significant differences were observed compared with the placebo group by post-hoc test, $0.01<^{*}p<0.05$. C5304, the C5304 bread-fed group, $n=17$; MoCA-J, Japanese version of the Montreal Cognitive Assessment; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=16$.

Table 7. Peripheral blood NK cells surface marker expression at baseline and post-intervention.

Expression (MFI)		Placebo	Y10M	C5304	*p*-value
NK CD69	Baseline	844.2±182.9	790.4±93.8	891.9±68.4	
	Post-intervention	791.1±214.9	842.1±103.3$^{\#}$	756.5±60.0	0.024
NK^{dim} CD69	Baseline	850.1±185.3	793.4±94.4	910.1±75.0	
	Post-intervention	800.1±222.9	847.2±105.9$^{\#}$	769.1±64.0	0.026

Data are presented as the mean±SE. All reported *p*-values were derived from ANCOVA models adjusted for baseline values, age, sex, and years of education, with Bonferroni correction applied to pairwise comparisons. Significant differences compared the Y10M group and the C5304 group by post-hoc test, $0.01<^{\#}p<0.05$. C5304, the C5304 bread-fed group, $n=17$; CD69, early NK cell activation marker; MFI, median fluorescence intensity; NK, natural killer cell; NK^{dim}, CD56^{dim} subset of NK cells; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=15$. In the Y10M group, one sample was excluded from NK activity measurement due to insufficient PBMC quality, resulting in a final sample size of $n=15$.

$CD3^{-}$ and $CD56^{+}$ phenotype. The CD56^{dim} subset of cells, also known as NK^{dim} cells, are mature NK cells.

Although both MFI and *Δ*MFI analyses revealed similar trends, slight differences were observed, potentially reflecting differences in baseline expression levels among the samples. Specifically, in MFI analysis the expression levels of CD69, an early NK cell activation marker, were significantly different between the Y10M and C5304 groups for both total NK cells ($p=0.024$) and NK^{dim} cells ($p=0.026$) (Table 7). The Y10M group showed significantly increased CD69 expression levels in total NK cells ($p=0.046$) and NK^{dim} cells ($p=0.043$) compared to the placebo group, and significantly increased CD69 expression levels in NK cells ($p=0.015$) and NK^{dim} cells ($p=0.014$) compared to the C5304 group (Fig. 4). No significant difference in CD69 expression was observed between the placebo and Y10M groups post-intervention; however, post-intervention values decreased compared to baseline in the placebo group but increased compared to baseline in the Y10M group. In addition, a significant positive correlation was observed between the MoCA-J total score and CD69 expression of NK cells ($p=0.027$) and NK^{dim} cells ($p=0.026$) (Fig. 5A, B). In the MoCA-J subscale "memory," although not statistically significant, a positive correlation trend was observed between CD69 expression of NK cells ($p=0.055$) and NK^{dim} cells ($p=0.052$) (Fig. 5C, D).

DISCUSSION

Overall, our results suggest that continuous consumption of maitake mushrooms is safe, as no adverse events that impeded daily life were observed, and the physical and hematological parameters remained within the standard range during the intervention over

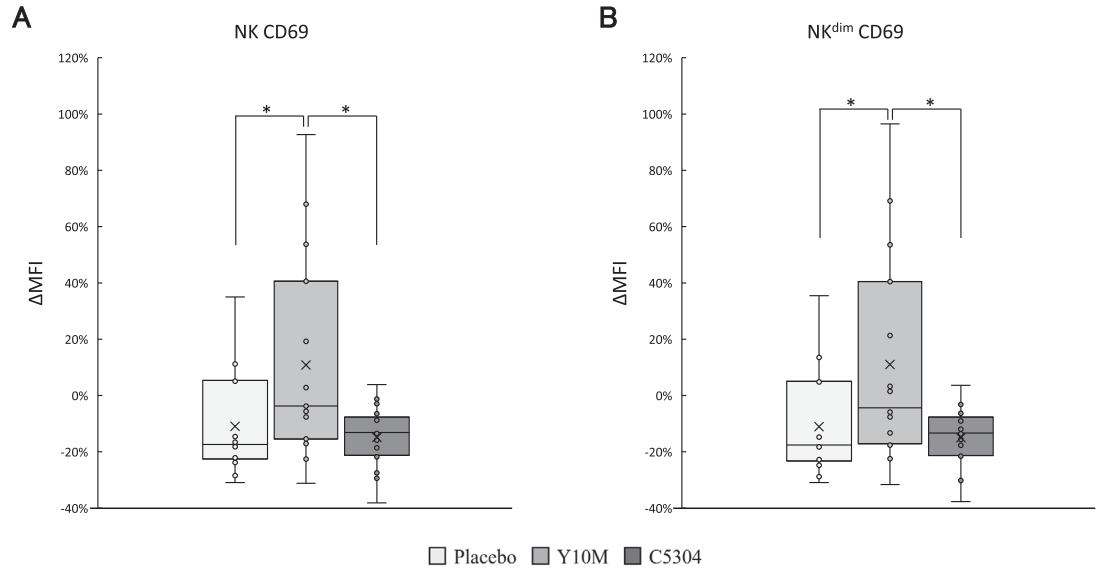


Fig. 4. Changes in the MFI of activated NK cells from baseline to 18 wk after the intervention. Changes in CD69 expression levels in (A) NK cells and (B) NK^{dim} cells from baseline to after 18 wk of intervention. The data are presented as box-and-whisker plots. The diagram and box boundaries are the same as Fig. 2. Statistical analyses were performed using ANCOVA adjusted for age, sex, and education (y). Significant differences were observed compared with the Y10M group by the post-hoc test, $0.01 \leqq * p < 0.05$. C5304, the C5304 bread-fed group, $n=17$; CD69, early NK cell activation marker; MFI, median fluorescence intensity; NK, natural killer cell; NK^{dim}, $CD56^{dim}$ subset of NK cells; Placebo, the group not fed either Y10M or C5304 bread, $n=14$; Y10M, the Y10M bread-fed group, $n=15$. In the Y10M group, one sample was excluded from NK activity measurement due to insufficient PBMC quality, resulting in a final sample size of $n=15$.

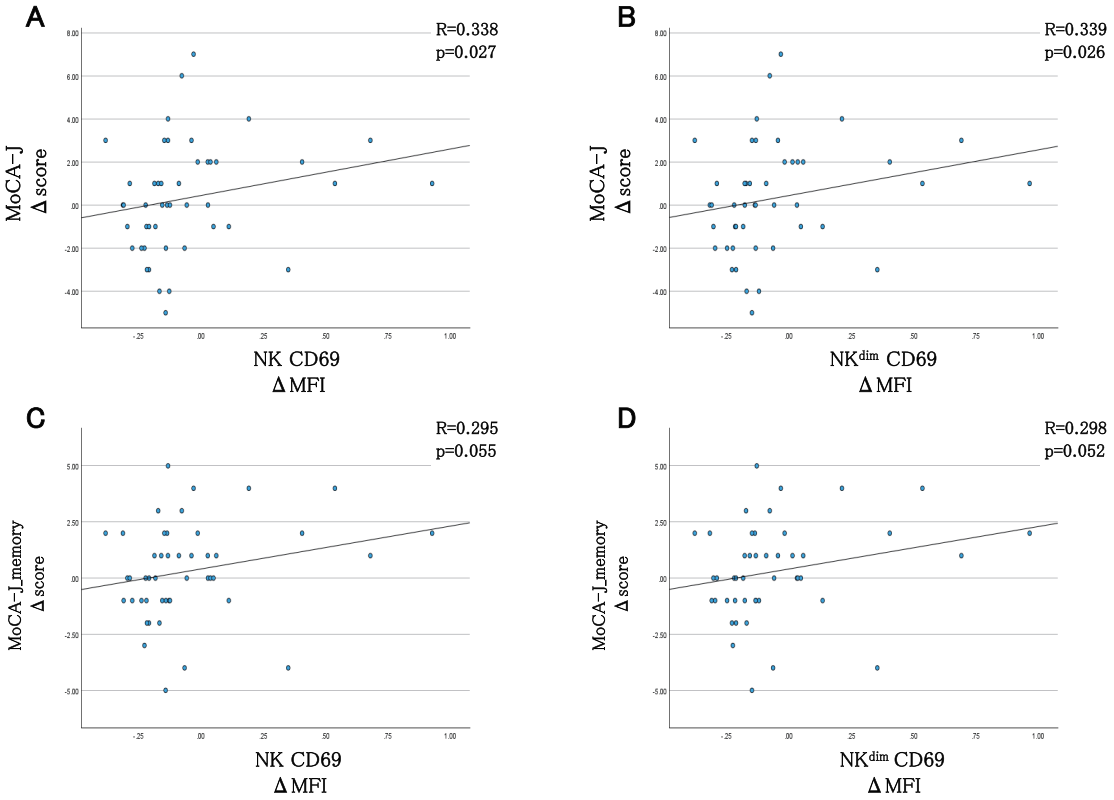


Fig. 5. Scatter plot of the relationship between changes in MoCA-J scores and NK cells activity. Relationship between MoCA-J total score and (A) NK cells and (B) NK^{dim} cells, and between MoCA-J subscale memory and (C) NK cells and (D) NK^{dim} cells. Statistical analyses were performed by using partial correlation coefficients adjusted for baseline values, age, sex, and education (y). CD69, early NK cell activation marker; MFI, median fluorescence intensity; MoCA-J, Japanese version of the Montreal Cognitive Assessment; NK, natural killer cell; NK^{dim}, $CD56^{dim}$ subset of NK cells. The plots represent all data from following groups: the Y10M bread-fed group ($n=15$), the C5304 bread-fed group ($n=17$), and the group not fed either Y10M or C5304 bread ($n=14$). In the Y10M bread-fed group, one sample was excluded from NK activity measurement due to insufficient PBMC quality, resulting in a final sample size of $n=15$.

18 wk (Table 2). The significant improvement of MoCA-J total score and subscale "memory" score in the Y10M group that consumed bread with maitake mushroom Y10M strain (Table 5) indicates that ingesting these mushrooms may be useful for maintaining cognitive function in healthy older individuals. Compliance with the intervention, that is, consumption of the daily dose of bread containing maitake mushrooms, was confirmed by an increase in blood ergosterol levels in the maitake groups after 18-wk. Ergosterol is a lipid found almost exclusively in fungi, including maitake mushrooms (*34*). Accordingly, the increased blood ergosterol levels observed in the maitake group appeared to have been induced by the intake and absorption of maitake mushrooms. To the best of our knowledge, this is the first randomized double-blind placebo-controlled trial to report the relationship between maitake mushroom consumption and cognitive function in healthy volunteers.

Rapid changes in the cognitive functions of healthy volunteers over the timeframe of this study were unlikely. Thus, of the three cognitive tests used, significant improvement was observed only in the MoCA-J, which is thought to have a high sensitivity for mild cognitive decline (Table 5). The MMSE is a simple and highly reliable test that is widely applied worldwide for dementia diagnosis; however, it has low sensitivity for the early symptoms of dementia and MCI and should be combined with other methods when used for early dementia screening (*35*). The HDS-R was developed in Japan in 1974 as a simple dementia diagnostic tool. Although it is widely used by clinicians in Japan, like the MMSE, the HDS-R is not considered reliable for detecting MCI (*31*). The MoCA, however, is a more complicated screening test that also evaluates executive function and visuospatial processing ability, and thus can detect small cognitive functional declines observed in early dementia. For example, the MoCA had the highest success rate in detecting MCI in a meta-analysis of 11 dementia-screening tools in 149 studies targeting patients with AD, Parkinson's disease, vascular dementia, and stroke (*36*). Furthermore, evaluation of the reliability of the MoCA and MMSE tests for MCI indicated that in subjects aged 60 y and older, the MoCA is more accurate than the MMSE in detecting MCI (*33*). Thus, the MoCA appears to be superior to the HDS-R and MMSE for detecting MCI. In the present study, a significant difference was observed for the MoCA-J subscale "memory." A study investigating MoCA-J characteristics in MCI and dementia cases reported that, of the six subscales in the MoCA-J, "memory" was a task with high difficulty (*37*). Thus, mild changes in cognitive function due to the consumption of maitake mushrooms may be best indicated by the subscale "memory." Taken together, these results suggest that consuming the maitake mushroom Y10M strain may contribute to maintaining cognitive function in healthy elderly individuals and might potentially help delay progression to MCI or dementia.

Our results are consistent with previous studies. For example, HDS-R scores increased significantly in 30 subjects with MCI who consumed the mushroom yamabushitake (*12*). In addition, cross-sectional studies have reported that mushroom consumption improves perceptual speed, executive function, semantic memory, and episodic memory in older individuals (*11*). A cohort study of 13,230 subjects investigating the relationship between mushroom consumption and dementia onset observed a significant inverse correlation between the frequency of mushroom consumption and risk of dementia onset (*10*). Cognition improved at 18 wk in this study, which is consistent with previous intervention reports showing that oral intake of foods or supplements for 6–24 wk also improved cognitive function (*6*, *38*). In the present study, the Y10M group showed a significant improvement in cognitive function compared with the placebo group after the 18-wk intervention, whereas no significant improvement was observed in the C5304 group. Previous studies have suggested that the health benefits and nutritional composition of mushrooms vary depending on the strain used. One study showed that the antioxidant activities of two *Auricularia auricular-judae* strains differed (*39*). Similarly, a report on the antioxidant activity and nutritional components of 16 *Pleurotus ostreatus* strains showed that each strain had different characteristics (*40*). These findings suggest that different mushroom strains have various health benefits. Maitake mushroom strains Y10M and C5304 show only slight differences in their nutritional components such as protein, fat, carbohydrates, and fiber (Table S1, Supplemental Online Material). However, their minor constituents and the structures of their nutrients such as polysaccharides remain poorly understood. Detailed characterization of these components may reveal the mechanisms underlying the functional differences between Y10M and C5304. Further research is required to investigate why the two maitake mushroom strains exhibited different effects in the present study.

NK cell activity was significantly augmented in the Y10M group (Fig. 4) and positively correlated with the MoCA-J score (Fig. 5), suggesting the involvement of the immune system in the improvement of cognitive function through the consumption of maitake mushrooms. Previous studies have reported on the immune-regulatory action of maitake mushroom. Consumption of maitake polysaccharides induced the proliferation of intestinal dendritic cells (DCs), macrophages, NK cells and T cells, regulating the systemic immunity anti-tumor effects (*16*). Several types of maitake mushroom extract induced DC maturation and activation, which promotes Th1 generation and subsequently enhances cellular immunity (*41*, *42*). It was shown that β-glucan rich maitake extract enhances NK cells activity (*26*). Although the mechanism of NK cells activation in this study is unclear, it may be attributable, at least in part, to polysaccharides contained in maitake such as β-glucan. In addition, administration of maitake mushroom-derived proteoglycans induced phagocytic activity in microglia, immune cells residing in the brain, thereby

improving cognitive function (*21*). These findings suggest that maitake mushrooms may contribute to the amelioration of neurodegenerative conditions by regulating the immune system.

Although the brain is thought to be isolated from systemic immunity, in recent years it has become clear that activation of systemic immunity plays an important role in maintaining brain function (*22*). Clinical studies have reported, for example, that inoculation with the bacillus Calmette-Guerin vaccine, the vaccine to prevent tuberculosis, inhibits the observed decreases in MoCA score (*43*) and reduces the risk of Aβ accumulation (*44*). Although many aspects related to the mechanism of vaccination-mediated improvements in cognitive function are unclear, possibilities include reduced risk of dementia due to prevention of infections, innate immune cell functional recovery due to epigenetic modifications, and brain tissue recovery due to nonspecific immune cell activation (*45*–*47*).

The presence of NK cells within the hippocampus has been debated due to its potential impact on various neurological disorders (*48*); however, it has also been demonstrated to mitigate intracerebral damage in Parkinson's disease and exert a neuroprotective effect (*49*). Moreover, NK cells effectively remove α-synuclein and potentially contribute to improve neurodegenerative conditions such as Lewy body dementia, and AD, which are characterized by α-synuclein deposition (*50*). It has also been reported that NK cell-deficient mice show impaired cognitive function (*51*) and NK cells induce Aβ clearance by improving microglial function (*23*). In addition, a phase 1 clinical trial utilizing NK cell treatment (SNK01) in patients with AD showed that 70% of patients exhibited stable or improved functional scores after the 11-wk intervention (*52*). In the present study, improvements in cognitive function and enhanced NK cell activity were confirmed in the Y10M group (Figs. 3 and 4), and there was a significant positive correlation between the MoCA-J score and NK cell activity (Fig. 5). These findings suggest that the ingestion of maitake mushrooms may promote the activation of immune cells, including NK cells, which weaken with age, thereby contributing, at least in part, to the maintenance and restoration of brain function.

The limitations of this interventional study include a restricted sample population and relatively small sample size, an unrestricted diet and intake method for any group, and no analysis of genetic backgrounds and lifestyle factors other than nutritional status. Therefore, latent confounding factors may not have been accounted for. Furthermore, the assessment of immune activity was limited to NK cells. In addition, it is unclear why the two maitake mushroom strains exhibited different effects. In the future, a larger-scale study that considers more detailed subject characteristics should be conducted, and the mechanism of cognitive function improvement resulting from the consumption of maitake mushrooms should be investigated further.

Although there is currently no established curative treatment for dementia, patients with MCI with normal cognitive function recovery have been reported (*5*). Therefore, early treatment of cognitive decline is important to combat dementia. The present study provides preliminary evidence that the daily consumption of maitake mushrooms may help attenuate cognitive decline in healthy older individuals. Incorporating maitake mushrooms into the daily diet could inhibit the shift from a healthy state to MCI, and from MCI to dementia, thereby playing an important role in maintaining healthy cognitive function.

Authorship

Conceptualization: MCK and MH; methodology: EJ, MCK, YM, MK, NK, SN, and SS; formal analysis: EJ, YM, and NK; investigation: EJ, MCK, YM, NK, KY, RA, MO, SK, and MH; resources: MO, SO, and SK; data curation: EJ, YM, and NK; writing—original draft preparation: EJ; writing—review and editing: MCK, MH, YM, MK, NK, and SN; visualization: EJ; supervision: MCK; project administration: MH; funding acquisition: EJ and MCK. All the authors have read and agreed to the published version of this manuscript.

Disclosure of state of COI

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Supporting information

Supplemental online material is available on J-STAGE.

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