

Infection-preventing composition, and food or drink containing the same

Field of the Invention

The present invention relates to an infection-preventing composition which comprises an extract of *Agaricus blazei* Murill and heat-treated cells of lactic acid bacteria, as effective ingredients, and a food or drink containing it.

Background of the Invention

Agaricus blazei Murill (Japanese generic name: "Kawari Haratake") which is a mushroom of *Agaricus* family of *basidiomycetes* has been known to show prevention and amelioration effects to cancer, allergy, diabetes, hypertension and the like. For Example, as an antitumor active ingredient, an acidic polysaccharide (JP-A-64-67194), a neutral polysaccharide (JP-A-64-67195) and a protein polysaccharide (JP-A-2-78630) are fractionated from its fruit body, a protein polysaccharide is fractionated from its mycelium (JP-A-61-47518), and a protein polysaccharide is fractionated from a culture filtrate of its mycelium (JP-A-61-47519).

Agaricus blazei Murill has been widely used as a material for health foods. For example, JP-A-2001-103927 discloses a food composition containing *Agaricus* mushroom extract which is obtained by extracting *Agaricus* mushroom with an aqueous solvent under raised pressure and fractionating the extract

effects of an extract of a fruit body of *Agaricus blazei* Murill, an extract of a culture of a mycelium of *Agaricus blazei* Murill, or a mixture thereof; and heat-treated cells of lactic acid bacteria.

In the above first aspect of the present invention, the lactic acid bacteria is preferably *Enterococcus faecalis*. Further, it is preferred that the extract of a fruit body of *Agaricus blazei* Murill, the extract of a culture of a mycelium of *Agaricus blazei* Murill, or the mixture thereof is contained in an amount of 0.5 to 99.5 mass%, and the heat-treated cells of lactic acid bacteria is contained in an amount of 99.5 to 0.5 mass%. According to the above embodiment, it is possible to provide an infection-preventing composition by which infection of pathogenic microbes and the like can be more effectively inhibited.

The second aspect of the present invention provides a food or drink containing the above infection-preventing composition.

In the above second aspect, it is preferred that the infection-preventing composition is contained in an amount of 0.01 to 10 g per portion.

The infection-preventing composition of the present invention contains as effective ingredients an extract of a fruit body of *Agaricus blazei* Murill and/or an extract of a culture of a mycelium of *Agaricus blazei* Murill conventionally ingested as foods, and heat-treated cells of lactic acid bacteria conventionally ingested as foods. Accordingly, the

safety of this composition is high. By ingesting it, excellent anti-infection effects to pathogenic microbes and the like can be expected. Further, by incorporating the infection-preventing composition of the present invention into food and drink, it is possible to provide food and drink by which infection of pathogenic microbes can be inhibited.

The mechanism about the effect of anti-infection by the infection-preventing composition of the present invention is not clearly known. However, it is believed that by ingesting both an extract of a fruit body of *Agaricus blazei* Murill, an extract of a culture of a mycelium of *Agaricus blazei* Murill, or a mixture thereof; and heat-treated cells of lactic acid bacteria in combination, cellular immunity mechanism mainly made of macrophages, NK-cells and T-cells can be activated, whereby elimination of pathogenic microbes can be effectively made.

Brief Description of the Drawings

Fig.1 is a graph showing survival rates of mice after infection with *Listeria monocytogenes*.

Fig.2 is a graph showing the results of measurement of the number of *Listeria monocytogenes* in spleens of mice after infection with *Listeria monocytogenes*.

Fig.3 is a graph showing the results of measurement of the cytokine amount (IL-2, IL-10, IL-12 and IFN- γ) in a supernatant of a culture solution of cells of mesenteric lymph nodes (MLN) by ELISA method.

Description of the Preferred Embodiments

The extract of a fruit body of *Agaricus blazei* Murill as one of effective ingredients of the infection-preventing composition of the present invention, is obtainable by extracting raw or dried fruit body of *Agaricus blazei* Murill with a solvent such as water or an alcohol. Specifically, for example, this is obtainable by adding water of about 20 times the mass of the fruit body of dried *Agaricus blazei* Murill, extracting it at 120°C for 30 minutes, and appropriately concentrating the obtained extract, followed by drying.

Further, the extract of a culture of a mycelium of *Agaricus blazei* Murill is obtainable by extracting in the same manner as above from a raw or dried material obtainable by culturing seed starter of *Agaricus blazei* Murill in a culture medium containing a carbon source and a nitrogen source.

In the present invention, as the extract of a fruit body, the extract of a culture of a mycelium of *Agaricus blazei* Murill, or the mixture thereof, commercially available ones may be used.

Further, the heat-treated cells of lactic acid bacteria as another effective ingredient of the infection-preventing composition of the present invention, is killed cells obtainable by heat treating lactic acid bacteria cells, and may for example be obtained as mentioned below.

Lactic acid bacteria are cultured in Rogosa medium at 30 to 45°C for 12 to 72 hours, and then the bacteria are collected by an appropriate means such as centrifugal separation. The

collected cells are washed with water, and the suspension of cells is concentrated, further the concentrated suspension of cells is heat treated at 80 to 115°C for 3 seconds to 30 minutes, and then dried by an appropriate means such as spray drying or freeze drying to obtain the desired cells. As the lactic acid bacteria, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus herveticus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Streptococcus thermophilus* and the like may be mentioned. In the present invention, it is particularly preferred to use *Enterococcus faecalis* which shows an extremely potent immunity-activating effect (ATCC 19433, ATCC 14508 and ATCC 23655). Further, the heat-treated cells of *Enterococcus faecalis* are commercially available as "EF Power" and "EF 2001" (both are tradenames, manufactured by Combi Corporation), "FK-23" (tradename, manufactured by Nichinichi Pharmaceutical), etc.

The infection-preventing composition of the present invention contains the extract of a fruit body of *Agaricus blazei* Murill, the extract of a culture of a mycelium of *Agaricus blazei* Murill, or the mixture thereof preferably in an amount of 0.5 to 99.5 mass%, more preferably 10 to 90 mass%, and the heat-treated cells of lactic acid bacteria preferably in an amount of 99.5 to 0.5 mass%, more preferably 90 to 10 mass%. If the amount of each effective ingredient is out of the above range, the desired synergistic infection-preventing effect tends to be low, such being undesirable.

The infection-preventing composition of the present invention may contain an excipient, a sweetener, an acidifier, vitamins, minerals, etc. in addition to the above essential components. Further, its configuration is not particularly limited, and powder, granules, tablets, capsules, liquid, etc. may suitably be selected depending upon the purposes.

Effective intake of the infection-preventing composition of the present invention is preferably 0.01 to 10 g, more preferably 0.05 to 2 g, per adult human a day.

The infection-preventing composition of the present invention may be added to various types of food and drink, such as drinks, jelly, candies, gum, retort foods (vacuum-packed foods), instant foods, etc. Added amount of the infection-preventing composition is preferably 0.01 to 10 g, more preferably 0.05 to 2 g, per portion. If the added amount of the infection-preventing composition is less than 0.01 g per portion, no sufficient infection-preventing effect can be obtained even if ingested, and if it exceeds 10 g per portion, the costs tend to be high, such being undesirable.

Examples

Hereinafter, the present invention will be explained in further detail with reference to examples. In the following examples, "Senseiro" (tradename, manufactured by Kyowa Engineering Co.) was used as an extract of a fruit body of *Agaricus blazei* Murill, and "EF 2001" (tradename, manufactured by Combi Corporation) was used as heat-treated cells of *Enterococcus faecalis*.

TEST EXAMPLE (Acute oral toxicity test of heat-treated cells of *Enterococcus faecalis*)

Acute oral toxicity test was carried out using mice in accordance with OECD Guidelines for the Chemicals 401 (1981).

Specifically, heat-treated cells of *Enterococcus faecalis* were suspended in purified water to prepare a test liquid (250 mg/ml).

After preliminarily breeding ICR series mice of 4 weeks old (20 mice of male and female, respectively; bought from Japan SLC, Inc.) for about one week, they were separated into Test group (10 mice of male and female, respectively) and Control group (10 mice of male and female, respectively). To the mice of Test group, the above test liquid was forcedly given by single oral administration with a gastric tube so that the test liquid amount would be 5,000 mg/kg of body weight in terms of heat-treated cells of *Enterococcus faecalis*. Further, to the mice of Control group, purified water (male: 0.7 ml, female: 0.6 ml) was administered similarly. The mice were bred in a breeding room set to have a temperature of $23 \pm 2^{\circ}\text{C}$ and lighting hours of 12 hours/day. During the test period, the mice were let to freely ingest water and feedstuff (tradename: "Solid feedstuff for mice and rats; Labo MR stock", manufactured by NIPPON NOSAN CO., INC.).

After the administration, observation was made once a day (observation period: 14 days), and no death was seen in both groups. After completion of the observation period, all the mice were subjected to autopsy, and no abnormality was seen in

main organs. Further, the weight was measured at the 7th day and 14th day after administration, comparison between the groups was made by t-test with a significance level of 5%. The results are indicated in Table 1.

Table 1

	Body weight (g)					
	Before administration		7th day after administration		14th day after administration	
	Male	Female	Male	Female	Male	Female
*1	30.7±1.2	26.7±1.5	34.9±1.4	29.2±1.9	37.3±1.9	31.0±1.8
*2	30.7±1.3	27.0±1.4	35.5±2.0	29.2±1.7	38.3±2.7	31.3±1.9

*1: Control group, *2: Test group

The body weight is indicated by "average value ± standard deviation (n=10)".

From Table 1, no difference is seen between the groups for both male and female. From the above results, it was considered that LD₅₀ value by single oral administration of the heat-treated cells of *Enterococcus faecalis* to mice is higher than 5,000 mg/kg of body weight.

Example 1

Forty BALB/c mice (female) of 7 weeks old (bought from CLEA JAPAN, INC.) were separated into four groups (10 mice in each group). To the mice of Test group, a mixture of an extract of a fruit body of *Agaricus blazei* Murill (0.3 mg/mouse in terms of a dried fruit body) and heat-treated cells of *Enterococcus faecalis* (10 µg/mouse) was given; to the mice of Comparative group 1, heat-treated cells of *Enterococcus faecalis* (20 µg/mouse) were given; to the mice of Comparative group 2, an extract of a fruit body of *Agaricus blazei* Murill (0.6mg/mouse

in terms of a dried fruit body) was given; and to Control group, PBS (0.2 ml/mouse) was given, respectively, by forced single oral administration with a gastric tube, once every day for one week.

Then, *Listeria monocytogenes* were injected into abdominal cavity of each of mice so that the injected amount would be 2×10^5 CFU/mouse, and the survival rates of mice of respective groups were inspected. During the test period, the mice were let to freely ingest water and feedstuff (tradename: "Powder Feedstuff CE-2", manufactured by CLEA JAPAN, INC.).

The results are shown in Fig.1. From Fig.1, Test group, Comparative group 1 and Comparative group 2 show extremely high survival rates as compared with Control group. Particularly, in Test group, no mouse was dead, and it is found that infection and proliferation of *Listeria monocytogenes* were prevented.

Example 2

In the same manner as in Example 1, once every day for one week before the start of test, test samples were given to mice of respective groups by forced oral administration, and then the mice were infected with *Listeria monocytogenes*. Then, spleens of mice were collected, and the change of number of *Listeria monocytogenes* in the infected spleens was measured. Specifically, after the infection, the spleens were collected at the 1st day, 3rd day, 5th day and 7th day, and the spleens were levigated and suspended in PBS. The suspensions were stepwise diluted, transferred onto "Enrichment Medium for *Listeria*" (tradename, manufactured by MERCK Co.), and cultured

(25°C, 48 hours), and then the number of emerged colonies was measured. The results are shown in Fig.2.

From Fig.2, it is found that at the 3rd day and thereafter after the infection, the number of *Listeria monocytogenes* increased in Control group, whereas the number decreased in Test group, Comparative groups 1 and 2. Particularly, the rate of decrease is large in Test group.

From these results, it is found that by orally ingesting an extract of a fruit body of *Agaricus blazei Murill* and heat-treated cells of lactic acid bacteria in combination, excellent infection-preventing effect can be shown as compared with the cases where each one is singly ingested.

Example 3

With respect to the action mechanism of infection-preventing effect by orally ingesting an extract of a fruit body of *Agaricus blazei Murill* and heat-treated cells of lactic acid bacteria in combination, studies were made by the following processes.

(1) Analysis of surface antigen of CD3-positive $\alpha\beta$ -type T-cells

After quarantine/receipt and inspection, six BALB/c mice (female, 7 weeks old; bought from Charles River Japan, Inc.) were preliminarily bred for one week, and then separated into two groups (3 mice per group). To the mice of Test group, a mixture (0.2 ml/mouse) of an extract of a fruit body of *Agaricus blazei Murill* (0.2 mg/mouse in terms of a dried fruit body) and heat-treated cells of *Enterococcus faecalis* (20 μ g/mouse) was

given; and to the mice of Control group, PBS (0.2 ml/mouse) was given, respectively, by oral administration with a gastric tube, once every day for one week. Then, *Listeria monocytogenes* were injected into caudal vein of each of mice so that the injected amount would be 1.4×10^4 CFU/mouse. During the breeding period, the mice were let to freely ingest water and feedstuff (tradename: "Powder Feedstuff CE-2", manufactured by CLEA JAPAN, INC.).

At the 5th day from inoculation of *Listeria monocytogenes*, the mice of respective groups were killed and their mesenteric lymph nodes were collected. The mesenteric lymph nodes were sandwiched between ground glasses, and suspended in a 10% FBS-containing RPMI-1640 culture solution (tradename, manufactured by Invitrogen Co.) for washing, and then excess tissues were removed by a stainless mesh to prepare cells of mesenteric lymph nodes (hereinafter referred to as MLN) of Test group and Control group, respectively.

Respective MLNs were suspended in a 10% FBS-containing RPMI-1640 culture solution so that the number of cells of MLN would be 5×10^5 /ml, and the suspension was sprinkled into a 6 well dish (manufactured by Corning Costar Co.) in an amount of 1 ml per well. As Stimulator, splenic cells originated from non-treated BALB/c mouse (5×10^6) and heat-killed cells of *Listeria monocytogenes* (1.0×10^6 CFU) which had been treated with mitomycin C (manufacture by KYOWA HAKKO KOGYO Co., Ltd.), were added to each well and mixed, and the mixture was cultured in the presence of a 5% CO₂ at 37°C for 48 hours. The above

untreated mouse means a mouse to which no administration of a test substance nor inoculation of bacteria was made.

Respective MLNs were stained with the following three types of labelled antibodies (all the antibodies were bought from Becton Dickinson PharMingen Co.), and analysis of surface antigen of cells was conducted.

(a) Cy-chrome(Cy) conjugated-anti CD3 ϵ mAb/FITC conjugated-anti TCR α β mAb/PE conjugated-anti CD4mAb/biotin conjugated-anti CD8mAb

(b) Cy conjugated-anti CD3 ϵ mAb/FITC conjugated-anti TCR α β mAb/PE conjugated-anti CD69mAb/biotin conjugated-anti CD8mAb

(c) Cy conjugated-anti CD3 ϵ mAb/FITC conjugated-anti TCR α β mAb/PE conjugated-anti CD122mAb/biotin conjugated-anti CD8mAb

Namely, to a MLN suspension adjusted to have 1.0×10^5 of cells of MLN, 5 μ l of labelled antibody (a) was added, followed by incubation at 4°C for 45 minutes. Then, washing with a 5% FBS-containing Hank's buffer solution was conducted twice, 5 μ l of SA-RED613 (tradename, manufactured by Invitrogen Co.) was added thereto, incubation was conducted at 4°C for 45 minutes, and washing with a 5% FBS-containing Hank's buffer solution was conducted three times. Then, the surface antigen of MLN was analyzed with a flow cytometer (model "Epics XL", manufactured by Beckman Coulter Inc.), to obtain the ratio of CD4⁺CD8⁺ cells to CD4⁺CD8⁻ cells and compare CD8-positive rates. The results are indicated in Table 2.

Table 2

	% of total CD3 ⁺ αβ TCR ⁺ cells		
	MLN (mean ± SD)		Ratio (B/A)
	CD4 ⁺ CD8 ⁻ (A)	CD4 ⁺ CD8 ⁺ (B)	
Control group	29.1±1.3	26.4±1.2	0.9±0.3
Test group	20.4±1.2	43.7±1.2	2.1±0.3

From Table 2, it is found that the CD8-positive rate of Test group is about 2 times higher than that of Control group and that the CD8-positive rate can be increased by ingesting an extract of a fruit body of *Agaricus blazei* Murill and heat-treated cells of *Enterococcus faecalis*.

Further, presence or absence of activation of CD8-positive T-cells was studied using CD69 (Present on activated T, B and NK lymphocytes) and CD122 (IL-2 receptor β-chain) as indicators. Namely, using the labeled antibodies (b) and (c), surface antigen of cells was analyzed in the same manner as above. The results are indicated in Table 3.

Table 3

	% of total CD3 ⁺ αβ TCR ⁺ CD8 ⁺ cells	
	MLN (mean ± SD)	
	CD69 ⁺	CD122 ⁺
Control group	38.6±1.2	35.5±1.8
Test group	42.2±1.2	43.8±1.4

From Table 3, it is suggested that the CD69- and CD122-positive rates of Test group are higher than those of Control group and that activation of CD8-positive T-cells can be accelerated by ingesting an extract of a fruit body of *Agaricus blazei* Murill and heat-treated cells of *Enterococcus faecalis*.

(2) Produced amount of cytokine

In the same manner as above, MLNs of Control group and

Test group were prepared, and Stimulator was added thereto, followed by culturing in the presence of a 5% CO₂ at 37°C for 48 hours.

After completion of culturing, supernatant of culture solution was collected, and the production of cytokines (IL-2, IL-10, IL-12 and IFN- γ) in the supernatant was measured by ELISA method. The measurements of IL-2, IL-12 and IFN- γ were made by a measurement kit bought from Endogen Inc., and the measurement of IL-10 was made by a measurement kit bought from AN' ALYZA TECHNE Co. The results are indicated in Fig.3

From Fig.3, it is found that in Test group, the production amounts of IFN- γ and IL-12 which are helper T-1 (Th-1) type cytokines are very high, and the production of IL-10 which is helper T-2 (Th-2) type cytokine is very low.

Further, production of cytokine-specific mRNA was researched by RT-PCR method, and it is found that in Test group, the expression of cytokine-specific mRNA of IL-12, IFN- γ and TNF- α which are Th-1 type cytokines were enhanced. On the other hand, no difference was seen in the production of IL-10 which is Th-2 type cytokine.

It is generally known that in living bodies infected with cytozoic bacteria such as *Listeria monocytogenes*, macrophages and NK-cells are activated, and these produce IL-12 and IFN- γ , respectively. These cytokines further activate bacterial antigen-specific CD8-positive T-cells, resulting in elimination of bacteria cells by CD8-positive T-cells having cytotoxicity activating function (cytotoxic lymphocyte).