# Immune Enhancing Effect by Orally-Administered Mixture of *Saccharomyces cerevisiae* and Fermented Rice Bran

## KOH, JONG HO, JIN MAN KIM[1], AND HYUNG JOO SUH[1]*

*Obesity Research Center, Dongduk Women's University, Seoul 136-174, Korea*
*[1]Department of Food and Nutrition, College of Health Sciences, Korea University, Seoul 136-703, Korea*

**Abstract** The mixture (PM) of *Saccharomyces cerevisiae* and fermented rice bran on the activation of macrophage and bone marrow cell proliferation was studied in mice. PM stimulated not only the activation of macrophage (1.8-fold of saline) but also IL-6 production from macrophage (1.5-fold) at 2.0 g/kg/day during 7 days of oral administration. By the culture supernatant of Peyer's patch cells from C3H/HeJ mice fed PM at 2.0 g/kg/day for 7 days, the bone marrow cells significantly proliferated compared with that of mice receiving only saline (1.7-fold). In addition, the contents of GM-CSF and IL-6 in the culture supernatant of Peyer's patch cells from mice fed PM at 2.0 g/kg/day were increased in comparison with those from the control (1.8 and 1.4-fold, respectively). These results revealed that oral administration of PM may modulate IL-6 production to induce the activation of macrophage, and also enhance secretion of hematopoietic growth factors such as GM-CSF and IL-6 from Peyer's patch cells.

**Key words:** Macrophage activation, Peyer's patch, *Saccharomyces cerevisiae*

In recent years, the feed manufacturing sector has promulgated the importance of functional feeds as dietary supplements for promoting health. Although probiotics represent a microbial feed supplement that beneficially influences the host by improving its intestinal microbial balance [3], it has to be expanded to different forms of administration and applications, and the concept of probiotics also defines the use of competitive exclusion for improving a specific ecology. These probiotics include a very large and constantly increasing group of microorganisms, and two groups of microorganisms, in general, are known as available probiotics. The largest group, comprised of lactic acid bacteria, has the ability to digest the lactose,

converting it into lactic acid and, therefore, lowering the micro-environmental pH. The other group is non-lactic acid bacteria such as *Bacillus* and *Saccharomyces*. Both groups have different mechanisms of activity, metabolism, and sensitivity to antibiotics [11]. Probiotics, including *Saccharomyces cerevisiae,* enhance the activity of beneficial microbes in the gastrointestinal tract, thus improving the digestibility of nutrients and production potential of the animals [7, 18, 22], whereas lactic acid bacteria probiotics such as *Lactobacillus* spp. competitively exclude the undesirable microorganisms from the intestine, thereby improving the health of the animal [17].

Claims of health benefits by probiotics should be clearly distinguished between resilience to disease and the psychological feeling of well-being. Proof of the former is difficult to demonstrate, because there have been no reports to date that convincingly proved that animals fed probiotics were resistant to experimental challenge with a pathogen. While the feeling of well-being is generally considered to be a psychosomatic parameter, there is increasing evidence that immunological criteria can be used to assess this condition. Environmental, physiological, social, and nutritional stressors inherent in intensive livestock animal production facilities have long been known to influence the growth performance and health. Stressors act on the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis, altering hormonal levels that in turn affect the production of cytokines by immune cells [2]. While acute stressors are known to stimulate the immunity, chronic stress is believed to down-regulate immune responses [15]. Use of immunological parameters to assess immune responses to dietary supplementation with various nutriceuticals or dietary and nutritional supplements may provide scientific validation of potential health benefits.

Therefore, the objectives of the present study were to assess the immunological enhancing effect as immunological parameter by oral administration of PM (the mixture of

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*\*Corresponding author*
Phone: 82-2-940-2853; Fax: 82-2-941-7825;
E-mail: suh1960@unitel.co.kr

*Saccharomyces cerevisiae* and fermented rice bran), and identify the possibilities of probiotics.

# MATERIALS AND METHODS

## Materials

RPMI-1640 medium and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY, U.S.A.). Heat-inactivated horse serum and fetal bovine serum were obtained from Cell Culture Laboratories (Cleveland, OH, U.S.A.), and penicillin, streptomycin, and amphotericin B were purchased from Flow Laboratories (Irine, Scotland). Alamar Blue™ was obtained from Alamar Bio-Sciences Inc. (Sacramento, CA, U.S.A.), and all other reagents were of analytical grade.

## Animals

C3H/HeJ (female, 5–7 weeks old) and ICR mice (male, 6–8 weeks old), and Sprague-Dawley rats (male, 6–8 weeks old) were purchased from Daihan-Biolink Co. (Korea) as experiment animals. Before adaptation, the animals were fed water *ad libitum* with a commercial chew pellet diet (Samyang Co., Korea), containing the following (g/kg diet) moisture, 80; protein, 230; fat, 35; fiber, 50; carbohydrate, 600.

## Feed Additives

Feed additives (YE, brewery yeast; PM, mixture of YE and SF; and SF, fermented rice bran), which were gifts from NEL Biotech. Co. Ltd. (Kyonggido, Korea), were used to measure the biological activities. YE is a dried form of brewery yeast (*Saccharomyces cerevisiae*) obtained as a byproduct during beer preparation. SF, a fermented rice bran, was prepared as follows; the growth medium contained 10% of rice bran, 0.6% of $(NH_4)_2SO_4$, 0.1% of $MgSO_4 \cdot 7H_2O$, 0.2% of $KH_2PO_4$, 0.03% of $K_2HPO_4$, and 0.1% of NaCl. *Saccharomyces cerevisiae* IFO 2346 and *Bacillus* sp. from soybean sauce were incubated in the above medium at 30°C for 5 days. After the incubation, SF contained the mixture of *Saccharomyces cerevisiae* (3× $10^5$ cfu/g) and *Bacillus* sp. (4×$10^6$ cfu/g).

## Macrophage Stimulating Activity

For determination of macrophage stimulating activity, 3 days before the experiment, 6-week-old mice, which had been orally administered with PM (the mixture of *Saccharomyces cerevisiae* and fermented rice bran) for 7 consecutive days at different doses, were injected aseptically with 1 ml of 3% thioglycollate broth via the intraperitoneal route. After peritoneal exudates, cells were harvested by the injection of 5 ml of the cold RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.) containing 5 mM HEPES, penicillin (100 U/ml) and streptomycin

(100 µg/ml) and they were adjusted to 1×$10^6$ cells/ml in RPMI-1640 medium. Samples were added and incubated at 37°C in a humidified atmosphere of 5% $CO_2$-95% air (48 h), and the macrophage stimulating activity was then estimated, using an assay system of the cellular lysosomal enzyme based on acid phosphatase activity produced by macrophages, by a microplate reader (Bio-Rad, Model 3550-UV) [4].

## Bone Marrow Cell Proliferation Activity Through Peyer's Patch Cells

Bone marrow cell proliferation activity was measured by the procedures of Hong *et al.* [6]. Peyer's patch cells of C3H/HeJ mice were suspended in RPMI-1640 medium supplemented with 5% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH, U.S.A.), and were stimulated with various concentrations of PM for 7 consecutive days by oral administration. After cultivation, the resulting culture supernatant (50 µl) was incubated with the bone marrow cell suspensions. After 20 µl of Alamar Blue™ (Alamar Bio-Science Inc., Sacramento, CA, U.S.A.) solution was added to the suspension, it was cultured [16], and the fluorescence intensity was measured to count the cell numbers by Spectrafluor Plus (Tecan, Austria) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm during cultivation.

## Assay of Cytokine Production

Murine IL-6 and granulocyte-macrophage-colony stimulating factor (GM-CSF) contents in the supernatant of macrophage and the cultures of Peyer's patch cells were measured by ELISA [1]. After a solution (2 µg/well and 10 µg/well) of purified anti-mouse IL-6 mAb and GM-CSF mAb (clone MP5-20F3 and MP1-22E9; PharMingen, San Diego, CA, U.S.A.) in 50 µl of bicarbonate buffer (pH 8.5) was adhered, unbound antibody was removed by washing 4 times with PBS containing 0.05% Tween 20 (PBS-Tween). Samples were first added to the antibody-coated well at 100 µl/well, and 100 µl of biotinylated anti-mouse IL-6 mAb and GM-CSF (MP5-32C11 and MP1-34D15, PharMingen) in PBS containing 10% fetal calf serum were added to the wells. After the plates were washed 6 times with PBS, 0.05% Tween 20 containing alkaline phosphatase labeled streptoavidin (Gibco, Grand Island, NY, U.S.A.) was added to each well. Each well was incubated with 150 µl of chromogenic substrate solution (1 mg of *p*-nitrophenyl disodium salt in 1 ml of 10% diethanolamine buffer, pH 9.8), and absorbance at 405 nm was subsequently measured using a microplate reader.

## Statistical Analysis

All results are expressed as the mean±S.D. The difference between the control and the treatments in these experiments was tested for statistical significance by Student's *t*-test. A

value of $P<0.05$ was considered to indicate statistical significance.

## RESULTS

### Immunological Activities of Feed Additives *In Vitro*

Several feed additives were investigated *in vitro* to find the ability of stimulating the macrophages. When compared with other feed additives, PM showed the highest macrophage activation, which was 2.0-fold more than the positive control, lipopolysaccharide (LPS) (Fig. 1A), while the

others showed lower activities than the control (LPS) even at a concentration of 500 mg/ml (data not shown). These results suggest that PM enhanced the stimulatory responses of macrophages. In addition, the effect of feed additives on Peyer's patch cells to mediate hematopoietic responses of bone marrow cells was investigated. As shown in Fig. 1B, when the conditioned medium of Peyer's patch cells obtained from C3H/HeJ mice, which were fed with PM, were added to the culture medium, the proliferation of bone marrow cells was significantly ($P<0.05$) different (1.8-fold increase) from that of mice that received water alone. These results indicated that PM is a potent inducer for hematopoietic growth factor.
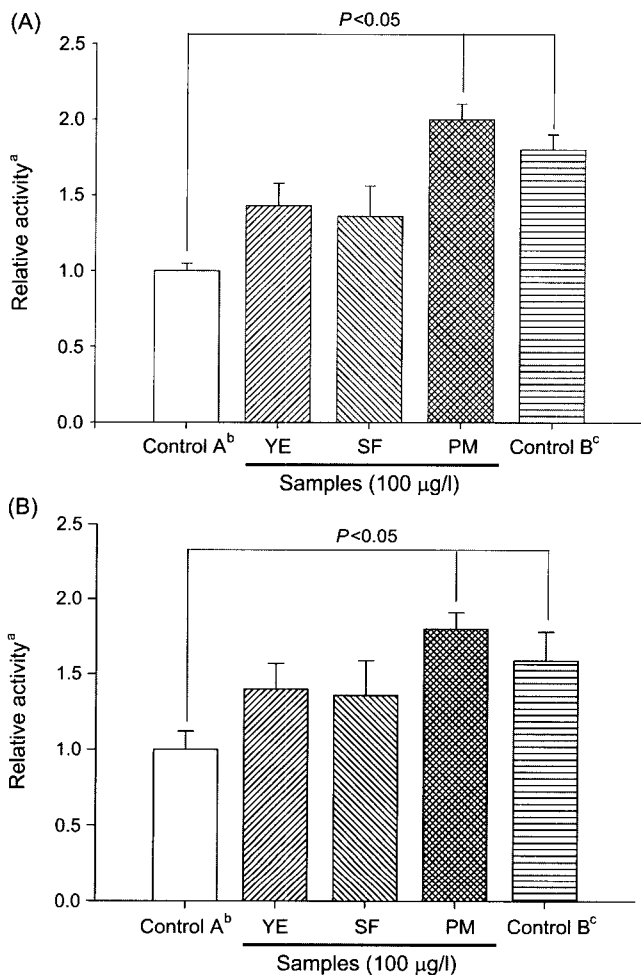


**Fig. 1.** A) Macrophage stimulating activity and B) bone marrow cell proliferation activity through Peyer's patch cells of feed additives *in vitro*.
[a]Relative activity; A) the ratio of enzyme activity of macrophage treated with feed additives to that of the control of macrophages from saline-treated mice; B) the ratio of fluorescence intensity of bone marrow cells treated feed additives to that of control of quadruplicate cultures. The final concentration of all samples was 100 μg/ml. Values represent mean (n=4)±S.D. $P<0.05$, Significance difference between control and samples. [b]Control A, saline without sample; [c]Control B, LPS as positive control. YE, *Saccharomyces cerevisiae*; SF, fermented rice bran; PM, the mixture of YE and SF.
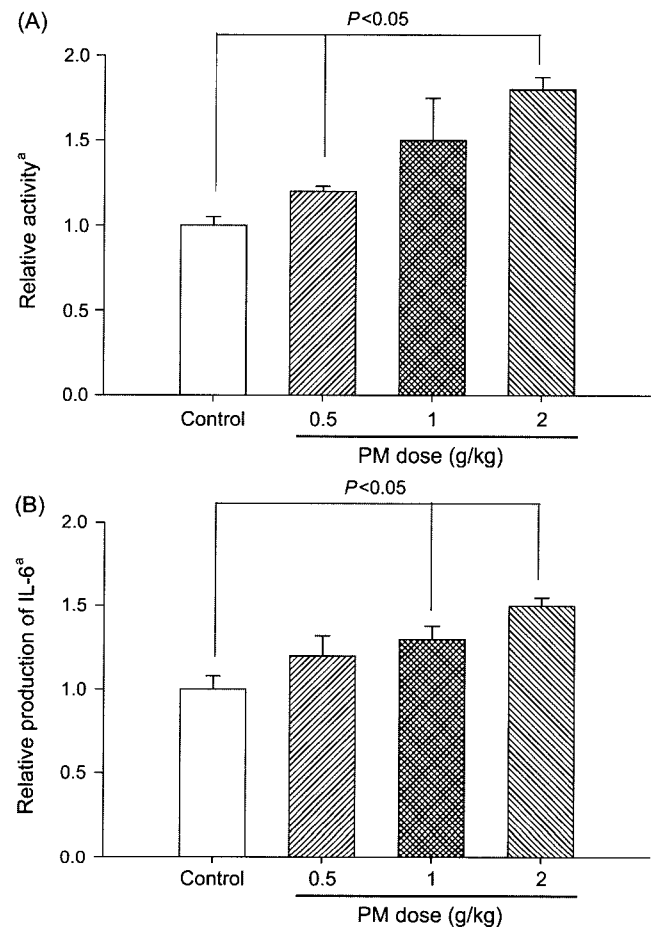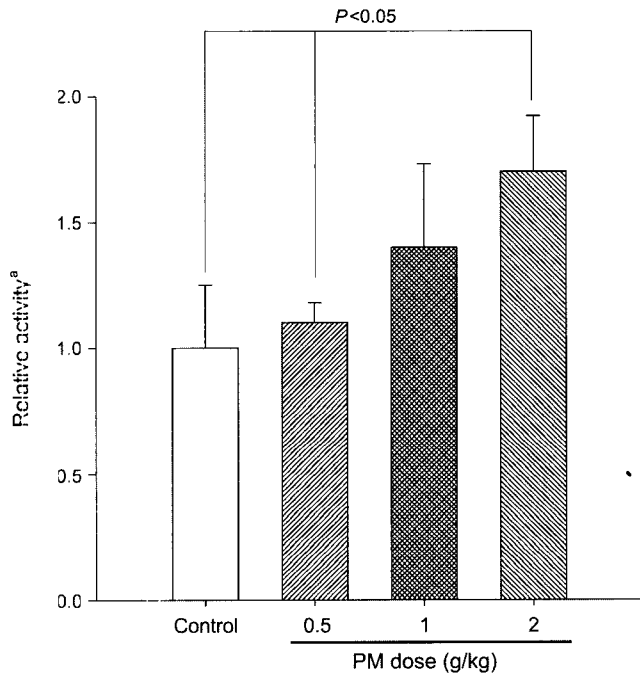


**Fig. 2.** A) Macrophage stimulating activity and B) IL-6 production in the culture supernatants of macrophages of ICR mice orally administered various concentrations of PM for 7 days.
Macrophage cells obtained from ICR mice (n=4) were orally administered PM at different doses (0.5, 1.0, and 2.0 g/kg per day), and were pooled and cultured for 2 days. The macrophage-stimulation (A) was measured using an assay system of the cellular lysosomal enzyme based on the activity of acid phosphatase from macrophages, and the resulting cell-free supernatants were subjected to ELISA for IL-6 production as described in Materials and Methods. Data was expressed as relative activity (A) and relative production of IL-6 (B) against control of quadruplicate assays. Values represent mean (n=4)±S.D. $P<0.05$, Significance difference between control and samples.

## Macrophage Stimulating Activity and Cytokine Production by Oral Administration of PM

To find PM's ability of stimulating macrophage in vivo, after the oral administration of different doses (0.5, 1.0, and 2.0 g/kg/day) of PM for 7 days, the stimulatory response of macrophage was measured by assaying cellular lysosomal enzyme activity, and the result is shown in Fig. 2. The administration of PM for 7 days resulted in a dose-dependent increase in the relative activity of macrophage lysosomal enzyme. A significant (P<0.05) increase in the relative activity was observed with the administration of 0.5 g/kg/day, and the maximum (1.8-fold) stimulation was observed with the administration of 2.0 g/kg/day (Fig. 2A), thus suggesting that the oral administration of PM enhanced the stimulatory responses of macrophages. In addition, the effects of orally-administered PM at different doses on IL-6 secretion from macrophages, which enhances IL-2 production from T cell and stimulates the proliferation of hematopoietic cells were also investigated in mice. Oral administration of PM was found to increase significantly and dose-dependently in comparison with the control, and PM stimulated the most IL-6 production at 2.0 g/kg/day (1.5-fold) (Fig 2B).

## Bone Marrow Cell Proliferation Activity Through Peyer's Patch Cells and Cytokine Production by Oral Administration of PM

The effect of PM on Peyer's patch cell-mediated hematopoietic responses of bone marrow cells was investigated. When the conditioned medium of Peyer's patch cells obtained from C3H/HeJ mice, which had been orally administered with different doses of PM for 7 consecutive days before the test, were added to the culture medium of bone marrow cells, the proliferation of bone marrow cells was significantly (P<0.05) enhanced compared with that of mice which received water alone. The resulting cell-free supernatant (conditioned media) of Peyer's patch cells from C3H/HeJ were administered for stimulation of bone marrow cells. As shown in Fig. 3, bone marrow cells
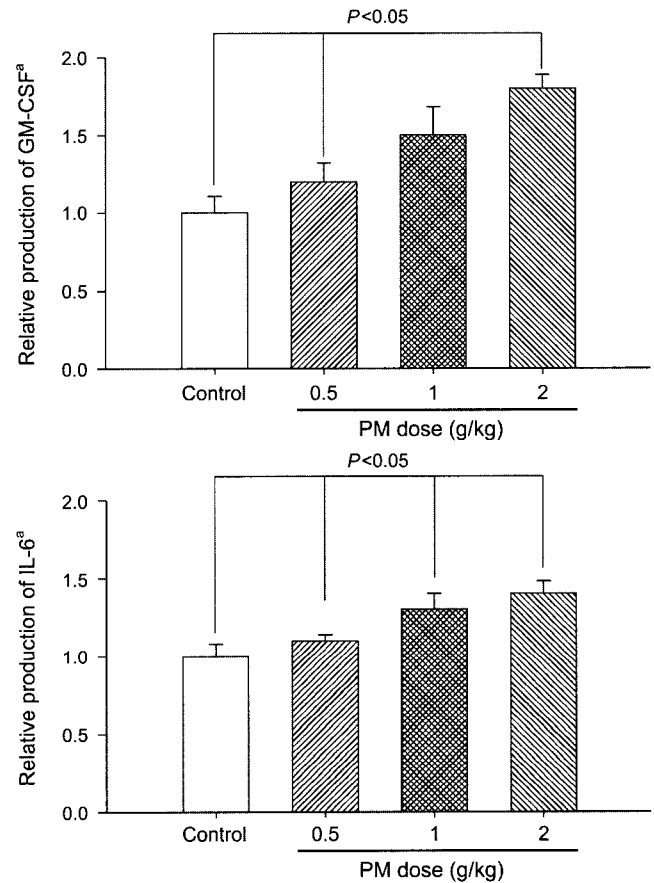


**Fig. 3.** Bone marrow cell proliferation activity through Peyer's patch cells of C3H/HeJ mice orally administered various concentrations of PM for 7 days.

Peyer's patch cells obtained from C3H/HeJ mice (n=4) were orally administered PM at different doses (0.5, 1.0, and 2.0 g/kg per day), and were pooled and cultured for 5 days. The resulting cell-free supernatant (conditioned medium) was subjected to stimulate bone marrow cells. The proliferation of bone marrow cells was measured by a fluorometric method using Alamar Blue™ reduction assay. [a]Relative activity, the ratio of fluorescence intensity of bone marrow cells treated feed additives to that of control. Data were expressed as mean±S.D. of quadruplicate cultures.



**Fig. 4.** Effect of orally administered PM on A) GM-CSF and B) IL-6 production in the culture supernatants (conditioned medium) of Peyer's patch cells.

Peyer's patch cells from treated mice (n=4) with PM at different doses per day for 7 days were pooled at a density of 4×10^{5} cells/well for 5 days in vitro. The resulting cell-free supernatants were subjected to ELISA for GM-CSF and IL-6 as described in Materials and Methods. [a]Relative production of GM-CSF and IL-6, the ration of absorbance of well reacted sample to that of control. Values represent mean (n=4)±S.D of quadruplicate assays. P<0.05, Significance difference between control and samples.

proliferated in a dose-dependent manner and almost reached plateau at over 2.0 g PM/kg per day. When 2.0 g PM/kg per day was used for the stimulation of Peyer's patch cells *in vivo*, the number of bone marrow cells increased up to 1.7-fold more than the control, as measured by Alamar Blue™ reduction assay. These results indicate that PM is a potent inducer for hematopoietic growth factor.

Lymphocytes, typically activated T cells, are known to secrete the growth factors such as GM-CSF and IL-6 [5], and these growth factors stimulate the proliferation of hematopoietic cells, followed by the differentiation into granulocytes or macrophages [23]. In order to find out whether PM induces GM-CSF and/or IL-6 secretion in Peyer's patch cells, Peyer's patch cells of C3H/HeJ, administered with different doses of PM for 7 days, were cultured for 5 days and then the conditioned medium of Peyer's patch cells were examined for GM-CSF and IL-6 activities. The GM-CSF content in the conditioned medium increased significantly ($P<0.05$) (1.8-fold of the control), when 2.0 g/kg per day was administered (Fig. 4A). IL-6 content also increased significantly ($P<0.05$) (1.4-fold of the control) in the conditioned medium when Peyer's patch cells were administered with 2.0 g/kg per day of PM (Fig. 4B). These results show that GM-CSF and IL-6 may, in part, contribute to the proliferation of bone marrow cells. Because both GM-CSF and IL-6 are multifunctional hematopoietic growth factors, they stimulate granulopoiesis [14].

## DISCUSSION

In recent years, health benefits from the consumption of probiotics have been used as a strong marketing tool, even though supporting data have not been available. The health effects of probiotic bacteria have recently been identified [8] and, in addition, many animal studies supported the evidence of probiotics to regulate immune responses *in vitro* [13, 21]. Lactic acid bacteria, particularly, have been studied for this activity, and it was reported that the mechanism to stimulate the immune response was due to the fact that the Gram-positive lipoteichoic acid was able to help antigens to bind to the epithelial cell membranes [20]. Interaction in cytokine production, mononuclear cell proliferation, macrophage phagocytosis, and killing has also been demonstrated, along with the modulation of autoimmunity and increase of specific immunity (IgA) [19, 10]. Recent studies on humans showed contrasting results, but if those health effects were also confirmed in humans, it may lead to applications in vaccination, and medical treatments for cancer, autoimmune disorders, and other diseases.

In the present study, PM as probiotics was shown to exert potent macrophage activation and bone marrow

cell proliferation through Peyer's patch cells in mice; particularly, enhancement of the cytokine production, such as IL-6, suggests that PM might induce the activation of macrophage. Therefore, PM is important for the killing of microorganisms in innate and adaptive immune responses, and should represent an immunopotentiator and biological response modifiers as probiotics. In addition, the effect of PM on bone marrow cell proliferation appears to be due to its ability to stimulate the production of IL-6 and GM-CSF from Peyer's patch cell. Since Peyer's patch cells are mainly composed of T and B cells, and T cells are known as the sources of CSFs and various cytokines as well as macrophages [12], T cell activation, caused by oral administration of PM, may contribute to the secretion of hematopoietic growth factors such as GM-CSF and IL-6 from Peyer's patch cells. Cytokines such as GM-CSF and IL-6 from Peyer's patch cells act on the cells and participate in the systemic immune system [9]. Therefore, it would be assumed that orally administered PM modulates not only the gastric mucosal immune system, but also the systemic immune system through a Peyer's patches' mediated mechanism.

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