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## Effect of fermented milk prepared with two probiotic strains on Japanese cedar pollinosis in a double-blind placebo-controlled clinical study

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## ABSTRACT

There has been much interest in the potential of using probiotic bacteria for treating allergic diseases. A double-blind, placebo-controlled study was conducted to examine the effectiveness of *Lactobacillus* GG (LGG) and *L. gasseri* TMC0356 (TMC0356) in alleviating Japanese cedar pollinosis (JCP), a seasonal allergic rhinitis caused by Japanese cedar pollen. Fermented milk prepared with the tested bacteria or placebo yoghurt was administered to 40 subjects with a clinical history of JCP for 10 weeks. Subjective symptoms, self-care measures and blood samples were compared between the two groups. Peripheral blood mononuclear cells (PBMCs) were collected from seven patients with JCP and *in vitro* cytokine production by the isolated PBMCs was analysed in the presence of heat-killed lactic acid bacteria. Consumption of the fermented milk significantly decreased the mean symptom score for nasal blockage after 9 weeks ( $P < 0.05$ ) and mean symptom-medication scores after 9 and 10 weeks when compared with the placebo group ( $P < 0.01$  and  $P < 0.05$  respectively). The tested strains of lactic acid bacteria affected cytokine production by isolated PBMCs *in vitro* in a strain-dependent manner. LGG significantly inhibited IL-4 and IL-5 production by PBMCs stimulated by both Cry j 1 and PHA. TMC0356 only suppressed IL-5 production stimulated by PHA. The fermented milk prepared with LGG and TMC0356 might be beneficial in JCP because of its effect on nasal blockage. The effects of LGG and TMC0356 might arise at least partly from their specific down-regulation of the human Th2 immune response.

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## 1. Introduction

Allergic diseases are becoming serious public health problems in Japan, as they are in many industrial countries. One such disease is Japanese cedar pollinosis (JCP), a seasonal allergic rhinitis caused by two major allergens (Cry j 1 and Cry j 2) in Japanese cedar (*Cryptomeria japonica*) pollen in spring (Yasueda et al., 1983; Sakaguchi et al., 1990). JCP is an IgE-mediated type I hypersensitivity, the main symptoms of which are sneezing, rhinorrhoea, nasal blockage and eye itching. In Japan, 16.2% of the population is estimated to be positive for IgE antibody specific to Japanese cedar pollen; the percentage affected is about 25% in 30- to 49-year-old people, who constitute the greater part of the working force in Japan (Okuda et al., 2005). JCP can impair the quality of life of both patients and their families, which reduces work productivity and school performance and hinders social activities. Anti-allergic medicines, such as anti-histamine agents and mast cell stabilizers, are available for the management of JCP. However, the side effects of the currently available medicines and the increasing cost of medicines has led to

great interest in the search for alternative solutions in the treatment and prevention of JCP.

Nasal blockage is one of the main symptoms in both the early and late phases of inflammation associated with allergic rhinitis. Sneezing and watery rhinorrhoea can be suppressed by antihistamine agents, which are widely used anti-allergic drugs. However, nasal blockage is not relieved by these anti-allergic drugs; it can only be relieved by  $\alpha$ -adrenoceptor stimulatory decongestants and corticosteroids, which induce tachyphylaxis when repeatedly used over a short period and other well-known side effects (Nabe et al., 2005). Therefore, a novel treatment for nasal blockage associated with allergic rhinitis is needed in the management of allergic rhinitis.

Although the specific mechanisms behind the epidemiological increase in the incidence of allergies such as JCP remains unclear, a hygienic environment with low microbial exposure and less diverse faecal and oral microbiota may provide less stimulation to the immune cells in the gut, favouring allergy-prone immunity and influencing the overall mucosal barrier (Strachan, 1989; Strachan, 2000; Rautava et al., 2004). The intestinal microbiota is a possible target in the management of allergic disorders, including allergic rhinitis (Björkstén et al., 1999; Björkstén, 2002; He et al., 2001). Probiotic bacteria may offer a safe and practical means of modulating

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the function and metabolic activity of the human intestinal microbiota and there has been much interest in the potential of using probiotic bacteria for treating allergic diseases. Recent clinical and animal studies have supported the hypothesis that lactobacilli, particularly certain selected strains with immunomodulatory properties, can modify the responses of the host, thereby inducing beneficial effects against allergic rhinitis (Ishida et al., 2005a,b; Morita et al., 2006; Kawase et al., 2006, 2007b). However, additional scientific evidence from well-designed human studies is necessary to demonstrate the effects of probiotics on JCP and the mechanisms underlying their actions. We performed a double-blind, placebo-controlled study to examine the potencies of *Lactobacillus* GG and *L. gasseri* TMC0356 in the form of fermented milk in alleviating JCP. Furthermore, these bacteria were tested for their effects on cytokine production *in vitro* by PBMCs isolated from patients with JCP.

## 2. Materials and methods

### 2.1. Bacterial strains

*Lactobacillus* GG (ATCC 53103) (LGG) was supplied by Valio (Helsinki, Finland). *Streptococcus thermophilus* TMC1551, *L. gasseri* TMC0356 (TMC0356) and *L. delbrueckii* subsp. *bulgaricus* TMC0222 were available at the Technical Research Laboratory of Takanashi Milk Products Co., Ltd. (Yokohama, Japan). Lactobacilli were routinely cultured at 37 °C for 18 h in MRS broth (Becton Dickinson, Sparks, MD, USA). *S. thermophilus* TMC1551 was routinely cultured at 37 °C for 18 h in Elliker broth (Becton Dickinson). After incubation, the bacteria were collected by centrifugation (7000 ×g) and washed three times with sterilized 0.85% NaCl. The washed bacteria were lyophilised and kept at –80 °C until use.

### 2.2. Subjects and cell preparation

Blood was collected from seven patients allergic to *C. japonica* pollen in January 2006, before the season of Japanese cedar pollen exposure (mainly from February to April). All volunteers were positive for IgE antibodies specific to Japanese cedar pollen (RAST class > 3). The clinical characteristics of the volunteers are shown in Table 1.

The collected heparinised peripheral blood was diluted in an equal volume of PBS. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (400 ×g, 35 min) over Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). After washing, cells were resuspended at  $2 \times 10^6$  cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (Gibco).

**Table 1**  
Clinical characteristics of subjects (co-activation assay of mononuclear cells)

| Characteristic                                                 |               |
|----------------------------------------------------------------|---------------|
| No. of subjects                                                | 7             |
| Men                                                            | 1             |
| Women                                                          | 6             |
| Age                                                            | 50.1 ± 5.7    |
| Specific IgE (score 0–6)                                       |               |
| Alder ( <i>Alnus japonica</i> )                                | 0.00 ± 0.00   |
| Japanese cedar ( <i>Cryptomeria japonica</i> )                 | 3.71 ± 0.29   |
| Japanese cypress ( <i>Chamaecyparis obtusa</i> )               | 2.00 ± 0.38   |
| Ragweed ( <i>Ambrosia artemisiifolia</i> var. <i>elatiar</i> ) | 1.14 ± 0.46   |
| Mugwort ( <i>Artemisia princeps</i> )                          | 0.14 ± 0.14   |
| Cock's foot grass ( <i>Dactylis glomerata</i> )                | 0.57 ± 0.43   |
| Mite ( <i>Dermatophagoides pteronyssinus</i> )                 | 1.71 ± 0.71   |
| House dust 1                                                   | 1.71 ± 0.71   |
| Dander (cat)                                                   | 0.43 ± 0.43   |
| Dander (dog)                                                   | 0.29 ± 0.18   |
| Total IgE                                                      | 520.0 ± 207.1 |
| Eos %                                                          | 4.37 ± 1.31   |

Means ± SE.

### 2.3. Stimulation of cytokine production *in vitro* by human peripheral blood mononuclear cells by lactic acid bacteria

Lactic acid bacteria (LAB) were heated at 100 °C for 30 min at  $2 \times 10^8$  cells/ml in PBS. To induce cytokine production, PBMCs ( $2 \times 10^6$  cells/ml) were incubated with heat killed LAB at a ratio of 10 bacteria per cell (10:1) and PHA (5.0 µg/ml; Serva Feinbiochemica, Heidelberg, Germany) or Cry j 1 (2.0 µg/ml), which was extracted and purified from *C. japonica* pollen as described previously (Yasueda et al., 1983) in 96-well microplates for 48 h at 37 °C in 5% CO<sub>2</sub>. The supernatants of the cell cultures were collected by centrifugation, and stored at –20 °C until use. IL-4, IL-5 and IFN-γ in the cell culture supernatant were analysed with commercial ELISA kits (BioSource International, CA, USA). The quantities of cytokines induced by the antigen in the presence of LAB were analysed statistically as percentages of control. Data were excluded from analysis when the cytokine concentration of the control was under the detection limit.

### 2.4. Preparation of fermented milk

Pasteurised milk (8.0% SNF, 2.0% milk fat) was inoculated with LGG and TMC0356, and incubated at 37 °C for 18 h (test fermented milk). The fermented milk had an acidity of 0.9% and bacterial counts of LGG and TMC0356 in the milk were  $> 1.4 \times 10^8$  cfu/ml and  $> 1.0 \times 10^7$  cfu/ml, respectively. Placebo yogurt was made by adding lactic acid to the same type of pasteurised milk as that used to make the test milk. The fermented milk and the placebo yogurt were not distinguishable from the package and sense of taste.

### 2.5. Subjects for clinical study

A total of 95 subjects with a clinical history of JCP were recruited from the area of Kanagawa prefecture for medical examination of the nasal cavity and blood clinical chemistry. After medical examination, 44 patients (15 males, 29 females; aged 20–57 years) were selected to participate in the study. Subjects who had milk allergy or perennial allergy rhinitis, or who were receiving specific immunotherapy or any drug treatment were excluded. Results on changes in immunological parameters for subjects taking cold medicines for more than 10% of the test period were excluded from the statistical analysis. All subjects lived in Kanagawa Prefecture, an area less than 40 km from the pollen-counting site (National Hospital Organization, Sagamiara National Hospital, Sagamiara-shi, Kanagawa). The study was conducted in accordance with the Declaration of Helsinki. All subjects were fully informed regarding the purpose and detailed procedure of experiment, and written informed consent was obtained. The study was approved by the Ethics Committee of Shinagawa East One Medical Clinic, Tokyo.

### 2.6. Clinical study design

Randomised double-blind and parallel studies were performed with the test fermented milk and the placebo. The clinical test was managed and operated by Dr S. Sugano of the Sugano Clinic of Ear Nose and Throat as an investigator, and was supported by Huma R&D, Tokyo, Japan, as a contract research organization. The trial was carried out for 10 weeks from 30 January to 11 April 2006. The 44 subjects were divided at random into two groups: group 1 received the fermented milk (22 subjects) and group 2 received the placebo yogurt (22 subjects). During the intervention period of 10 weeks, the subjects consumed 110 g of fermented milk or placebo every day.

### 2.7. Patient monitoring

Nasal symptoms (sneezing, rhinorrhoea, blockage and itching), eye symptoms (itching, redness and weeping) and throat symptoms

(itching and redness) were recorded separately. Patients evaluated their symptom scores on a five-point scale defined by the Japanese Society for Allergy: 0, absent (no symptoms); 1, mild; 2, moderate; 3, severe; 4, very severe (Okuda et al., 2005). The patients were permitted to use medication for allergic symptoms, and daily records were made of the use of medicines (times, name of medicine and purpose of medication). Patients recorded the medication score on a three-point scale defined by the Japanese Society for Allergy (Okuda et al., 2005). The sum of the symptom score and medication score was used as the symptom-medication score. These scores were evaluated as weekly averages.

## 2.8. Medical examination and blood sampling

Medical examinations (physical examination, blood pressure and nasal examination) were conducted and blood samples were taken three times: before and after 6 and 10 weeks of consumption of the test products. On nasal examination, the investigator diagnosed four nasal symptoms of the patients (swelling and colour of the inferior turbinate, and amount and appearance of nasal discharge). Patients evaluated their symptoms on a four-point scale defined by the Japanese Society for Allergy (Okuda et al., 2005).

Blood tests were conducted to measure total IgE, anti-Japanese cedar pollen IgE, Th1/Th2 ratio, thymus and activation-regulated chemokine (TARC), C-reactive protein, eosinophils, total protein, albumin, total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, urea nitrogen, creatinine, uric acid, total bilirubin, glutamic-oxaloacetic transaminase, glutamate-pyruvate transaminase,  $\gamma$ -glutamic transpeptidase, alkaline phosphatase, lactate dehydrogenase, cholinesterase, glucose, white blood cells, red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, Na, K, Ca and Cl. TARC was measured at Mitsubishi Chemical BCL, Tokyo, Japan. The Th1/Th2 ratio was measured at SRL, Tokyo, Japan. The other measurements were performed at the Health Science Research Institute, Yokohama, Japan.

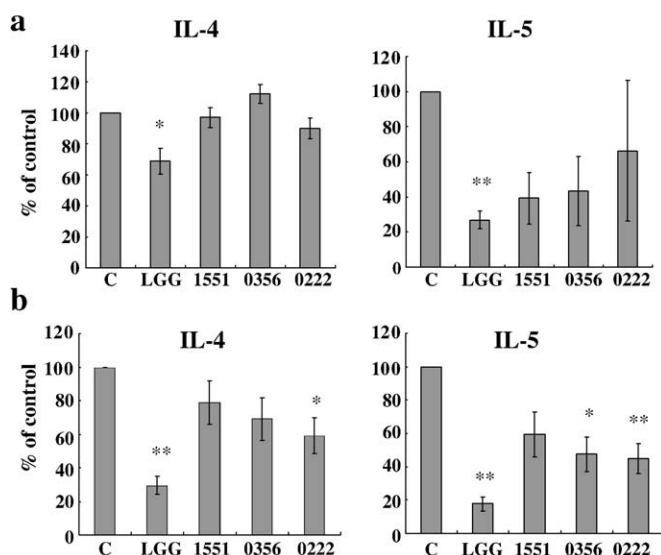


**Fig. 1.** Effects of LAB on IL-4 and IL-5 production by antigen-stimulated PBMCs. (a) Cry j 1 (b) PHA. PBMCs were incubated with heat-killed LAB (LGG, *Lactobacillus* GG; 1551, *Streptococcus thermophilus* TMC1551; 0356, *L. gasseri* TMC0356; 0222, *L. delbrueckii* subsp. *bulgaricus* TMC0222). IL-4 and IL-5 in the supernatant were measured by ELISA. Data are mean  $\pm$  SE (IL-4 production with PHA and Cry j 1,  $n=7$ ; IL-5 production with PHA,  $n=6$ ; IL-5 production with Cry j 1,  $n=5$ ). \*\* $P<0.01$ ; \* $P<0.05$ , significantly different from control (C).

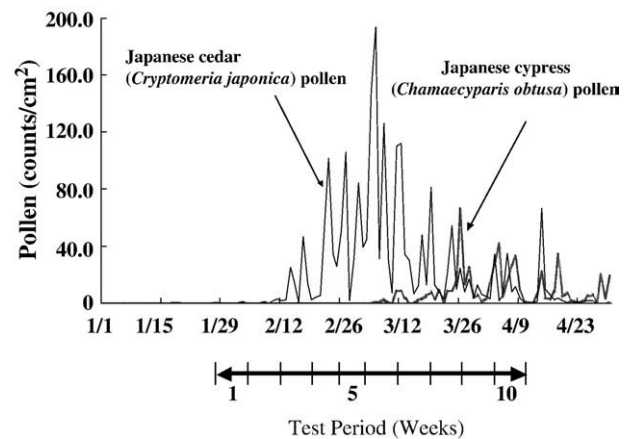


**Fig. 2.** Japanese cedar pollen counts during the 2006 season in Sagami-hara City area.

## 2.9. Statistical analysis

In the coactivation assay of mononuclear cells, the statistical significance of differences between control and test groups was evaluated using the two-tailed multiple  $t$ -test with Bonferroni correction (four comparisons in five groups). In the clinical study, the statistical significances of difference between the two groups were calculated using the Mann-Whitney  $U$  test for symptom score and symptom-medication score, and unpaired Student's  $t$ -test was used for blood examination results. Data were analysed using SPSS (version 14.0) for Windows, SPSS Japan Inc., Tokyo, Japan).

## 3. Results

### 3.1. Effects of lactic acid bacteria on cytokine production from antigen-stimulated peripheral blood mononuclear cells

PBMCs isolated from allergic volunteers to Japanese cedar pollen were cultured with heat-killed LAB in the presence of Cry j 1 or PHA (Fig. 1). Quantities of cytokines induced by the antigen varied widely among the volunteers, so the quantities of cytokines induced by the antigen in the presence of LAB were analysed statistically as percentages of control. LGG decreased IL-4 and IL-5 production induced by Cry j 1 in comparison with the control (IL-4,  $P<0.05$ ; IL-5,  $P<0.01$ ). IL-5 production induced by Cry j 1 decreased in two treatment groups of *S. thermophilus* TMC1551 and TMC0356. However such differences were not statistically significant. IL-4 production induced by PHA was decreased markedly in cocultures with LGG ( $P<0.01$ ). A significant decrease was observed not only with LGG but also with *L. bulgaricus* TMC0222. IL-5 production induced by PHA was decreased in three treatment groups of LGG, TMC0356 and *L. bulgaricus* TMC0222 ( $P<0.01$ ,  $P<0.05$ ). IFN- $\gamma$  production was not seen when Cry j 1 or PHA was added.

### 3.2. Pollen count during period of clinical test

Fig. 2 shows the daily averages of the Japanese cedar pollen and Japanese cypress (*Chamaecyparis obtusa*) pollen counts during the 2006 season in the Sagami-hara City area. Average Japanese cedar pollen counts for weeks 1, 6 and 10 of the clinical test were 0.3, 87.4 and 14.2/cm<sup>2</sup> respectively.

### 3.3. Baseline characteristics of patients

Baseline characteristics were similar in the placebo group and the group receiving the test milk (prepared with LGG and TMC0356) (Table 2). Nineteen of 22 patients in the placebo and 21 of 22 patients in the group receiving the test milk completed the 10-week study

**Table 2**  
Clinical characteristics of subjects (clinical test)

| Characteristic                                   | Placebo   | LGG–TMC0356 |
|--------------------------------------------------|-----------|-------------|
| No. of subjects                                  | 19        | 21          |
| Men                                              | 6         | 6           |
| Women                                            | 13        | 15          |
| Age                                              | 36.8±1.3  | 36.9±1.7    |
| Height (cm)                                      | 163.2±2.1 | 163.0±1.9   |
| Body weight (kg)                                 | 57.8±2.1  | 57.5±3.3    |
| BMI                                              | 21.6±0.5  | 21.4±0.8    |
| Specific IgE (score 0–6)                         |           |             |
| Milk                                             | 0         | 0           |
| Japanese cedar ( <i>Cryptomeria japonica</i> )   | 3.42±0.15 | 3.33±0.13   |
| Japanese cypress ( <i>Chamaecyparis obtusa</i> ) | 1.42±0.18 | 1.38±0.18   |
| Alder ( <i>Alnus japonica</i> )                  | 0.58±0.17 | 0.05±0.13   |
| House dust                                       | 1.47±0.18 | 0.95±0.21   |
| Mite ( <i>Dermatophagoides farinae</i> )         | 1.37±0.18 | 1.10±0.21   |

Data are expressed as the mean±SE.

(Fig. 3). Discontinuation was for private reasons. One of 19 patients in the placebo group and one of 21 patients in the LGG–TMC0356 group took cold medicines for more than 10% of the test period and were therefore excluded from statistical analysis of the immunological results.

### 3.4. Clinical effects of lactic acid bacteria on subjective symptoms

The effect of orally administered fermented milk prepared with LGG and TMC0356 was examined with respect to changes in scores for subjective symptoms and medication during a 10-week period. Fig. 4 shows the changes in symptom score (Fig. 4a) and symptom-medication score (Fig. 4b) for nasal blockage in each group. The symptom score and symptom-medication score of both groups increased after the increase in pollen count. Mean symptom score for nasal blockage in the LGG–TMC0356 group after 9 weeks of the test was significantly lower than that for the placebo group ( $P<0.05$ ). That for the LGG–TMC0356 group after 10 weeks of the test was lower than that for the placebo group. However such difference was not statistically significant ( $P=0.053$ ). Mean symptom-medication scores for nasal blockage in the LGG–TMC0356 group at 9 and 10 weeks were significantly lower than those for the placebo group ( $P<0.01$  and  $P<0.05$  respectively). However, there was no significant difference between the two groups in the symptom scores for sneezing, rhinorrhoea and itching (Fig. 5).

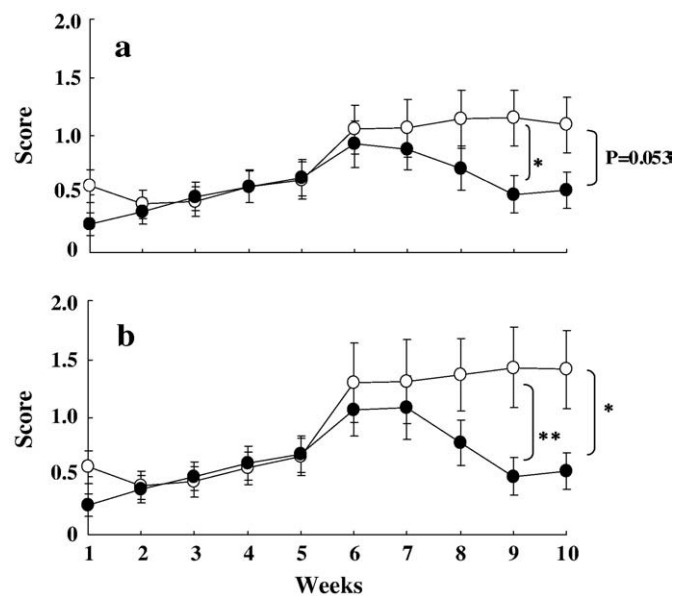


**Fig. 4.** Symptom scores (a) and symptom-medication scores (b) for nasal blockage in subjects receiving fermented milk prepared with LGG and TMC0356 (closed circles) and placebo yogurt (open circles). Points represent weekly means of daily values and bars represent the standard errors (LGG–TMC0356,  $n=20$ ; placebo,  $n=18$ ). \*\* $P<0.01$ ; \* $P<0.05$ .

### 3.5. Blood examination and medical examination of nasal cavity

Levels of TARC and eosinophils increased in both groups following the increase in pollen count (Table 3). However, there was no

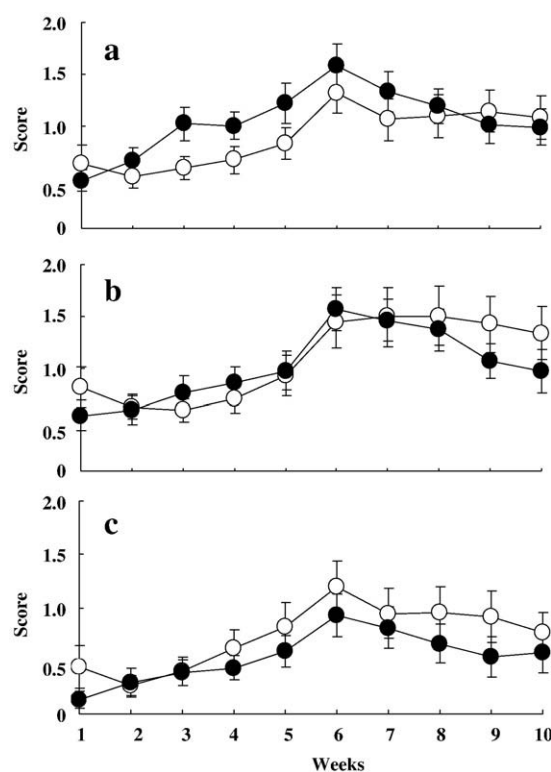


**Fig. 5.** Symptom scores for (a) sneezing, (b) rhinorrhoea and (c) itching for groups receiving fermented milk prepared with LGG and TMC0356 (closed circles) and placebo yogurt (open circles). Points represent weekly means of daily values and bars represent the standard errors (LGG–TMC0356,  $n=20$ ; placebo,  $n=18$ ).

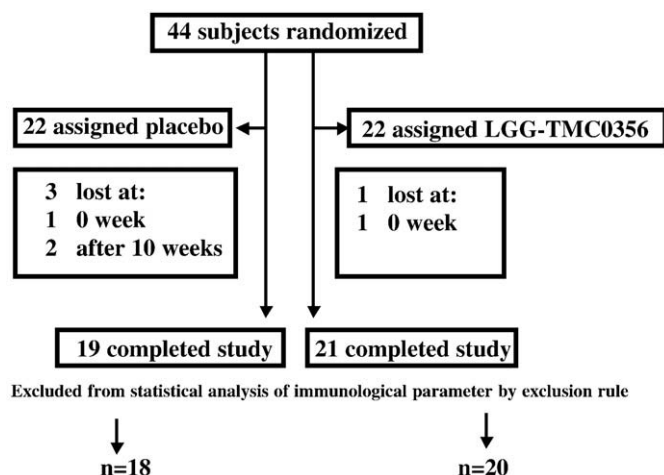


**Fig. 3.** Trial profile.

**Table 3**  
Immunological parameters in blood

| Item                                   |             | Weeks       |             |             |
|----------------------------------------|-------------|-------------|-------------|-------------|
|                                        |             | 0           | 6           | 10          |
| Total IgE (IU/ml)                      | Placebo     | 107.9±24.6  | 111.7±27.0  | 124.2±31.1  |
|                                        | LGG–TMC0356 | 104.7±21.9  | 113.7±25.4  | 123.1±32.9  |
| Anti-Japanese cedar pollen IgE (UA/ml) | Placebo     | 17.2±3.6    | 14.9±3.1    | 18.7±4.1    |
|                                        | LGG–TMC0356 | 18.1±4.0    | 19.0±4.8    | 22.5±7.0    |
| Th1/Th2                                | Placebo     | 15.3±1.0    | 16.6±2.2    | 18.7±2.2    |
|                                        | LGG–TMC0356 | 17.5±2.0    | 13.6±1.7    | 17.0±1.9    |
| TARC (pg/ml)                           | Placebo     | 303.6±37.0  | 357.1±41.4  | 295.4±31.2  |
|                                        | LGG–TMC0356 | 263.0±19.7  | 366.3±27.9  | 296.4±19.9  |
| CRP (mg/dl)                            | Placebo     | 0.151±0.073 | 0.063±0.027 | 0.086±0.027 |
|                                        | LGG–TMC0356 | 0.087±0.027 | 0.096±0.044 | 0.038±0.011 |
| Eosinophils (cells/ $\mu$ l)           | Placebo     | 213.1±43.0  | 248.9±39.8  | 237.2±39.6  |
|                                        | LGG–TMC0356 | 138.3±27.0  | 247.5±33.3  | 194.0±37.7  |

Each value represents the mean and the standard error (placebo,  $n=18$ ; LGG–TMC0356,  $n=20$ ).

significant difference between the two groups in these measurements. There was no change in anti-Japanese cedar pollen IgE following the increase in pollen count. Results for some biochemical and haematological blood variables changed after the increase in pollen count but remained within normal ranges (data not shown).

Four nasal symptoms of the patients (swelling and colour of the inferior turbinate, and the amount and appearance of nasal discharge) were evaluated at 0, 6 and 10 weeks. However, there was no significant difference between the two groups in any of the nasal symptoms (data not shown).

#### 4. Discussion

*Lactobacillus* GG (ATCC 53103) was originally isolated from the human intestine and is used considerably in probiotic preparations. This bacterium reduced the clinical symptoms of atopic eczema in nursing infants whose mothers were given LGG and in milk-hypersensitive infants who consumed a hydrolysed whey formula supplement with LGG (Majamaa and Isolauri, 1997). A 7-year follow-up clinical study demonstrated that this probiotic bacterium can effectively protect against the development of atopic disease in infants with a high genetic risk of allergic diseases (Kalliomäki et al., 2001, 2003, 2007). LGG also improved atopic eczema/dermatitis in other well-designed clinical studies and the stimulation of IFN- $\gamma$  production was suggested as a possible mechanism for these health-promoting effects (Pohjavuori et al., 2004; Viljanen et al., 2005). However, the effect of *Lactobacillus* GG on birch pollen allergy has not been observed in a clinical study targeting teenagers and young adults (Helin et al., 2002). No clinical evidence to support the use of LGG in JCP is available. Recently, several selected lactobacilli and bifidobacteria have been evaluated for their possible ability to alleviate JCP in animal and human studies (Shida et al., 1998; Ishida et al., 2003, 2005a; Tamura et al., 2007). However, the clinical effects of these bacteria on JCP in humans were not impressive and were inconsistent with those expected from animal studies. Allergic disorders that occur in adults, such as JCP, might be relatively difficult to manage because of the heterogeneity of the genetic background affecting allergic sensitization and the various factors influencing symptom development, such as lifestyle, daily behaviour and living environment. The intestinal microbiota of adults is more complicated than that of infants, which might reduce the effects of probiotics on aspects of the human immune system that are involved in allergies. Therefore, probiotic regimens targeting JCP need to be modified. The combination of different probiotic strains has recently been suggested as a practical means of improving the potency of probiotic therapies in the treatment of allergic disorders.

TMC0356 was originally isolated from healthy adult intestine. It activates macrophages to secrete pro-inflammatory (IL-12) and anti-

inflammatory (IL-10) cytokines (Morita et al., 2002). This bacterium suppresses the increase in serum IgE concentration that occurs in mice and human subjects with perennial allergic rhinitis (Morita et al., 2006; Kawase et al., 2007a). However, LGG did not mediate serum IgE synthesis in allergic subjects, although the bacterium showed a slight effect on serum IgE concentration in OVA-sensitized mice (Kalliomäki et al., 2001). On the other hand, NC/Nga mice fed LGG showed a significant increase in the plasma level of IL-10 and increased IL-10 mRNA expression in both Peyer's patches and mesenteric lymph nodes (Sawada et al., 2007). These results suggest that TMC0356 and LGG can alter host immunity in a strain-dependent manner. TMC0356 was able to normalize Th2-dominated allergic immunity by enhancement of the host's Th1 immune response, and LGG might be able to improve allergic symptoms through its anti-inflammatory effects. The combination of these bacteria may be expected to enhance immune-regulatory effects in the host animal. Indeed, a combination of LGG and TMC0356 significantly suppressed antigen-induced nasal vascular permeability and alleviated antigen-induced nasal blockage in rats and guinea-pigs (Kawase et al., 2006, 2007b). The present double-blind, placebo-controlled clinical study was performed in the light of these previous studies in order to evaluate the clinical effects of a combination of LGG and TMC0356 in JCP.

In the present study, the level of TARC in both the test and the placebo group showed a close relationship with the level of cedar pollen from January to April 2006, indicating that allergic inflammation had been induced by exposure to cedar pollen, although the density of cedar pollen in 2006 was less than usual. The symptom score for nasal blockage in the test group decreased from 6 weeks of intervention, when the scattering of pollen count reached its peak, and was suppressed significantly after 9 weeks of the intervention. These results indicate that the combination of LGG and TMC0356 can alleviate allergic symptoms in the nose, especially nasal blockage, as did these bacteria in animal studies. However, we did not observe these improvements of allergic symptoms until 6 weeks after the start of administration of the bacteria. In several recent clinical studies with other strains, the benefits of probiotics in JCP were limited to ocular symptoms and no significant effects were observed on nasal symptoms (Ishida et al., 2005a,b; Xiao et al., 2006). Therefore, the present study might be the first evidence for the possibility that probiotics can alleviate nasal blockage associated with JCP in both animal and human studies (Kawase et al., 2007b). However, no significant improvement was observed in sneezing, rhinorrhoea and itching in the present study. More innovation is needed in order to expand the spectrum of probiotic therapy by adding new strains, both qualitatively and quantitatively. Our results suggest that different probiotic strains can improve the allergic symptoms involved in JCP in different ways, and that the combination of LGG and TMC0356 can alleviate the nasal blockage associated with allergic rhinitis. The results also suggest that certain probiotic strains might be used as a potent novel treatment for the nasal blockage associated with allergic rhinitis.

The proteins in the cedar pollens designated Cry j 1 and Cry j 2 are major antigens for cedar pollinosis, although some additional antigenic proteins have been found recently in Japanese cedar pollen (Yasueda et al., 1983; Sakaguchi et al., 1990; Fujimura et al., 2007). Patients with Japanese cedar pollinosis possess IgE antibodies against Cry j 1 or Cry j 2, or even both of these proteins (Sakaguchi et al., 1990; Hashimoto et al., 1995). The high Th2 immune response caused by Cry j 1 and Cry j 2 is considered to be a key underlying mechanism for the high level of IgE synthesis in JCP patients, and a prospective target in the management of JCP. In the present study, the production of IL-4 and IL-5 was significantly stimulated by Cry j 1 from PBMCs isolated from JCP patients, whereas the production of IFN- $\gamma$  was not modified. These results confirm again that Cry j 1 can act as a strong stimulator of Th2 immunity in JCP patients. LGG significantly inhibited the production of IL-4 and IL-5 by PBMCs, stimulated by Cry j 1. It also

inhibited IL-4 and IL-5 production from isolated PBMCs induced by PHA. Whereas, TMC0356 only suppressed PHA-stimulated IL-5 production by the tested PBMCs, as did other tested bacteria. Although TMC0356 did not downregulate Cry j 1-induced IL-4 production in this study, TMC0356 reduced Th2 cytokine (IL-4, IL-5) production and increased Th1 cytokine (IFN- $\gamma$ ) production by splenocytes from OVA-sensitized mice (Kawase et al., 2007a). The different actions of TMC0356 in mice splenocytes and human PBMCs may be explained by the fact that the phenotype of CD4<sup>+</sup> T cells in humans is not differentiated as clearly as in mice. Considering the above results, it may be that the effects of LGG and TMC0356 on nasal blockage in the present study are at least partly a result of the effects of these bacteria on the Th2-type allergic immune response, especially IL-5-related chronic inflammation. Birch and timothy pollen *in vitro* induced IFN- $\gamma$  and IL-4 mRNA expression by PBMCs in allergic patients, while Cry j 1 did not mediate IFN- $\gamma$  production by PBMCs isolated from JCP patients (Laaksonen et al., 2003). These results suggest that allergy to birch and timothy pollens might differ from JCP, and that the way in which LGG improves JCP may differ from the way in which it improves birch pollen allergy.

No significant changes in serum IgE and other biomarkers in the blood related to IgE immunity were observed in the present study after the intervention. Similar observations have been made in several animal studies as well as human studies. LGG and TMC0356 significantly inhibited the OVA-stimulated nasal vascular permeability of rats and improved the OVA-induced nasal blockage of guinea-pigs (Kawase et al., 2006, 2007b). However, total serum IgE in these animals was not significantly altered. Therefore, the association between improvement in allergic symptoms and alteration in IgE synthesis remains unclear. Recently, the allergen-induced synthesis of IL-5, and not of IgE, has been shown to be a key mechanism linked to symptomatic episodes of seasonal allergic rhinitis in sensitized individuals (Ohashi et al., 1998). IL-5 seems to be a hallmark of the clinical manifestation of seasonal allergic rhinitis caused by Japanese cedar pollens (Nakai et al., 2000). Therefore, a therapeutic strategy to target allergen-induced IL-5 could be recommended for the control of allergic symptoms in individuals.

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