

# A Double-blind Trial of *Lactobacillus paracasei* Strain KW3110 Administration for Immunomodulation in Patients with Pollen Allergy

Daisuke Fujiwara<sup>1</sup>, Hideyuki Wakabayashi<sup>1</sup>, Hiroyuki Watanabe<sup>1</sup>, Satoshi Nishida<sup>2</sup> and Hisakazu Iino<sup>3</sup>

## ABSTRACT

**Background:** *Lactobacillus paracasei* strain KW3110 has been shown by *in vitro* cytokine secretion analysis to be a potent Th1 inducer and Th2 repressor. Oral administration of *L. paracasei* strain KW3110 also represses IgE elevation in the ovalbumin sensitized mouse allergy model.

**Methods:** During pollen season from January to April in 2003, a daily dose of  $4 \times 10^{10}$  cfu *L. paracasei* strain KW3110 was fed for 12 weeks as a yogurt to 14 pollen allergy patients (mean age 39.0 years). A yogurt made with *Lactobacillus delbrueckii* strain B, shown to be a poor Th1 inducer and Th2 repressor in a former study, was also fed at a daily dose of  $4 \times 10^{10}$  cfu for 12 weeks to 14 pollen allergy patients (mean age, 39.0 years). Blood samples were collected every 4 weeks. Eosinophils, eosinophil cationic protein (ECP), non-specific serum IgE, cedar pollen specific serum IgE, and Th1/Th2 ratios were quantified.

**Results:** Twelve weeks after ingestion of the yogurts, the group that was fed *L. delbrueckii* group showed significant reductions in the Th1/Th2 ratios between the first observation in January and last observation in April, representing the four month study period, due to increases in Th2 cells. Significant increases in ECP were also observed in the group fed *L. delbrueckii* between January and April. In contrast, the group fed *L. paracasei* had no significant changes in the ECP and Th1/Th2 ratios.

**Conclusions:** Our results suggest that ingestion of the *L. paracasei* strain KW3110 is associated with both repression of Th2 cell generation and eosinophil activation. Our data point to the possibility that specific lactic acid bacteria may be useful for allergy therapy.

## KEY WORDS

ECP, *Lactobacillus paracasei* strain KW3110, Th1, Th2, yogurt

## INTRODUCTION

There is growing interest in the anti-allergic effect of lactic acid bacteria (LAB). We have recently shown that LAB strains vary widely in the ability to improve the consequences of the Th1/Th2 imbalance. *L. paracasei* strain KW3110 was identified as a potent inducer of IL-12 and repressor of IL-4 in a study of 101 strains that were tested *in vitro* using ovalbumin (OVA) sensitized Th2-polarized mice splenocytes.<sup>1</sup> Some studies indicate that clinical symptoms and in-

flammation markers for allergy are alleviated after administration of probiotic bacteria.<sup>2-5</sup> The mechanisms of efficacy of the probiotic bacteria may be mediated by both immune stimulation and improvement of composition of the intestinal flora. The proof of an inverse association between bacterial infections and allergy has led to the "hygiene hypothesis" <sup>6</sup> that suggests the recent rapid rise in allergy and autoimmune diseases is a result of improved hygiene and reduced bacterial infections.<sup>7</sup> Therefore, a corollary of the hygiene hypothesis would be that allergic diseases im-

<sup>1</sup>Central Laboratories for Key Technology, Kirin Brewery Co., Ltd.,

<sup>2</sup>Technical Development Center, Koivai Dairy Products Co., Ltd. and <sup>3</sup>Department of Science for Living System, Showa Women's University, Kanagawa, Japan.

Correspondence: Daisuke Fujiwara Ph.D., Central Laboratories

for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawa, Yokohama, Kanagawa 236-0004, Japan.

Email: d-fujiwara@kirin.co.jp

Received 22 April 2004. Accepted for publication 30 July 2004.

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**Table 1** Characteristics of patients enrolled in the study

|                                | <i>L. paracasei</i> | <i>L. delbrueckii</i> | <i>P</i> value |
|--------------------------------|---------------------|-----------------------|----------------|
| <i>n</i>                       | 14                  | 14                    | —              |
| Age (y)                        | 39.0                | 39.0                  | 0.95           |
| Sex (male : female)            | 8 : 6               | 5 : 9                 | —              |
| sIgE (UA/ml)                   | 15.6                | 9.8                   | 0.34           |
| tIgE (IU/ml)                   | 241.0               | 123.4                 | 0.37           |
| Eosinophils (counts / $\mu$ l) | 154.3               | 194.3                 | 0.46           |
| ECP ( $\mu$ g/L)               | 13.5                | 15.6                  | 0.46           |
| Th1/Th2                        | 12.0                | 14.0                  | 0.41           |
| Th1 (%)                        | 20.1                | 26.8                  | 0.07           |
| Th2 (%)                        | 2.4                 | 2.2                   | 0.66           |

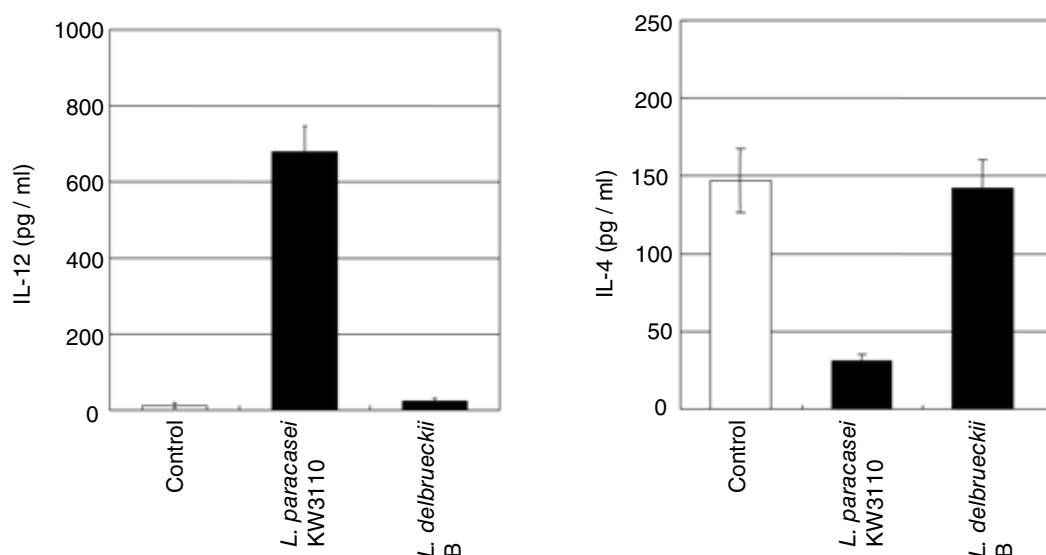


**Fig. 1** Comparison of immune stimulating activities between *L. paracasei* strain KW3110 and *L. delbrueckii* strain B on OVA-sensitized BALB/c splenocytes. Secretion of IL-12 and IL-4 from OVA sensitized BALB / c splenocytes was determined by ELISA. A concentration of 1  $\mu$ g / ml of each LAB strain was added to each culture and 7 days culture supernatants were analyzed. Each bar represents the mean  $\pm$  SD of 6 mice.

prove by administration of some microbes. LAB are non-pathogenic, gram-positive bacteria that should be candidates for treatment of allergic diseases by oral administration.

In Japan, one fourth of the population suffers from allergy that is primarily caused by cedar pollen. The allergy season in Japan occurs between the winter to spring seasons during the natural dispersal of the cedar pollen. We have conducted a double-blind clinical trial to compare the effect of feeding yogurts manufactured by either *L. paracasei* strain KW3110 or *Lactobacillus delbrueckii* strain B. Serum allergy markers<sup>8-10</sup> in study participants were compared in the groups ingesting the two different yogurts during the major pollen allergy season in Japan.

## METHODS

### IN VITRO CYTOKINE SECRETION IN MICE SPLENOCYTES

OVA-primed splenocytes were used for *in vitro* studies. Female 6 week old BALB/c mice (Charles River, Yokohama, Japan) were injected intraperitoneally with 1 mg of OVA absorbed onto 2 mg of Al(OH)<sub>3</sub> (Wako, Tokyo, Japan). Injections were given twice on day 0 and on day 6 post initial immunization. On day 13 mice were sacrificed to obtain splenocytes. After lysis of RBC, splenocytes were suspended at the concentration of  $2 \times 10^6$  cells/ml in RPMI medium (Sigma, MO, USA) supplemented with 10% FCS and penicillin/streptomycin. Cells were cultured for 7 days with 1 mg/ml OVA, in either the presence or absence of 1

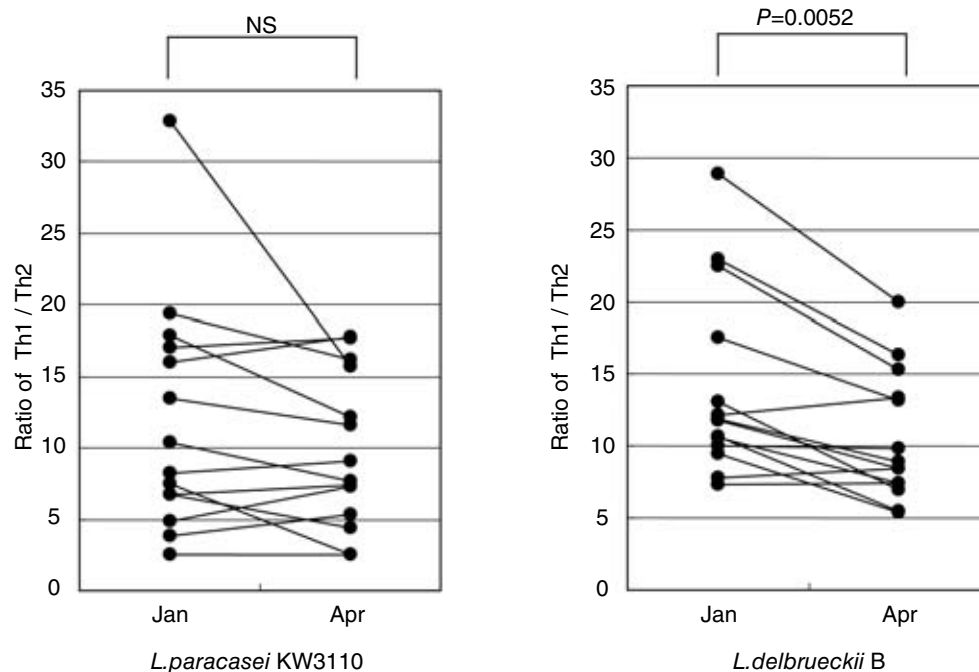


**Fig. 2** Th1 / Th2 ratios in individual study participants before (Jan) and after (Apr) supplementation for 12 weeks with yogurt manufactured by either *L. paracasei* strain KW3110 or *L. delbrueckii* strain B. *P* values are shown in brackets at the top of each panel. NS = not significant ( $P > 0.05$ )

µg/ml of bacteria.

### BACTERIAL STRAINS

*L. paracasei* strain KW3110 and *L. delbrueckii* strain B were maintained at Koiwai Dairy Products Co., Ltd. For the mouse cytokine secretion assay, bacteria were grown at 37°C for 48 hr in MRS broth (DIFCO, MI, USA). Cultured bacteria were washed twice with sterile distilled water, heat-killed at 100°C, lyophilized, and suspended in PBS.

### SUBJECTS

Twenty-eight adult patients who suffered from cedar pollen allergy participated in the study. All patients were employed by Kirin Brewery Co., Ltd and Koiwai Dairy Products Co., Ltd. Cedar pollen-specific IgE-positive ( $>0.34$  UA/ml) patients were selected as subjects. Patients were allowed to use drugs only when they displayed severe symptoms. There was one patient for each group who had used magistral drugs. The study was conducted according to the ethical standards of our company, which required informed consent from each patient.

### STUDY DESIGN

A double-blind design was used. Subjects were randomly divided into 2 groups of 14 patients per group. The 12-week study began in January 2003, before the pollen season, and ended after pollen season, in April 2003. The yogurts produced by the 2 strains of bacte-

ria (*L. delbrueckii* strain B and *L. paracasei* strain KW3110) were fed individually to participants in each group. The characteristics of patients of both groups when this study had begun are shown in Table 1. No significant difference was observed between *L. paracasei* strain KW3110 group and *L. delbrueckii* strain B group in all parameters. However, there was a trend for the *L. delbrueckii* strain B group to be higher than *L. paracasei* strain KW3110 group in Th1 cells ( $P = 0.07$ ). Each subject was given 200 ml yogurt daily for 12 weeks. Both types of yogurt were manufactured by Koiwai Dairy Foods, Co., Ltd and contained about  $2 \times 10^8$  cfu/ml.

### QUANTITATION OF EOSINOPHILS AND SERUM ECP

Heparinized blood samples were collected every 4 weeks and serum was stored at -80°C. ECP measurements were performed from 4 ml blood samples. ECP levels were measured with Uni-Cap ECP kit and an automated fluoroenzyme assay analyzer (Uni-Cap 100) following the manufacturer's instructions (Pharmacia Diagnostics, Uppsala, Sweden).

### SERUM IgE MEASUREMENT

Commercial kits (Pharmacia Diagnostics) were used to measure both total serum IgE (Uni-Cap total IgE) and cedar pollen specific IgE (Uni-Cap Japanese cedar specific IgE) using the Uni-Cap 100 automated analysis.

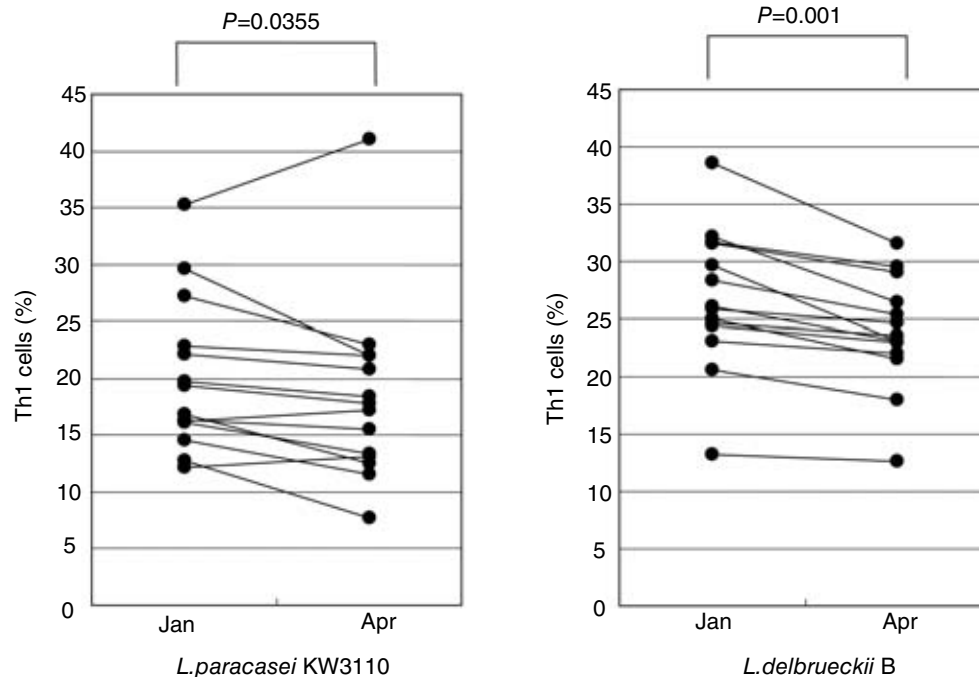


**Fig. 3** Percentages of Th1 cells in individual participants before (Jan) and after (Apr) supplementation for 12 weeks with yogurt manufactured by either *L. paracasei* strain KW3110 or *L. delbrueckii* strain B.

### TH1/TH2 RATIO MEASUREMENT

500  $\mu$ l of each blood sample were incubated at 37°C for 4 hrs in 500  $\mu$ l RPMI-1640 (Sigma) supplemented with 12.5  $\mu$ g of phorbol 12-myristate 13-acetate (Sigma), 1  $\mu$ g of ionomycin (Sigma) and 5  $\mu$ g of brefeldin A (Sigma). Then cells were collected and stained with APC-conjugated mouse anti human CD4 (Beckman Coulter, MI, USA). Cells were fixed for 15 min at room temperature in FACS lysing solution (Becton Dickinson, CA, USA). Cells were treated with FACS permeabilizing solution 2 (Becton Dickinson) according to the manufacturer's instructions. Cells were stained for IFN- $\gamma$  to quantify Th1 cells and IL-4 to quantify Th2 cells with FASTIMMUNE IFN- $\gamma$  FITC/IL-4 PE (Becton Dickinson) for 30 min on ice, washed in dPBS/BSA, and stored in the dark at 4°C. Samples were analyzed on a Becton Dickinson FACS caliber flow cytometer using the CellQuest software package. Live gating on lymphocytes or CD4<sup>+</sup> events was performed during acquisition. The CD4<sup>+</sup>, IFN- $\gamma$ <sup>+</sup> population and the CD4<sup>+</sup>, IL-4<sup>+</sup> population were defined as Th1 and Th2 cells, respectively. The Th1/Th2 ratio was calculated by dividing the percentage of the Th1 population by the percentage of Th2 population.

### STATISTICS

The serum parameter data were analyzed by Wilcoxon's signed rank test when comparing the data before and after the cedar pollen season and by the

Mann-Whitney *U*-test when compared the data of both groups. The differences in treatment are given as the mean with 95% confidence intervals. A *P* value less than or equal to 0.05 was considered to indicate a statistically significant difference.

## RESULTS

### IN VITRO EFFECTS OF TH1/TH2 BALANCE IMPROVEMENT IN MICE

The ability of the LAB strains being studied to induce the Th1 cytokine IL-12 and the Th2 cytokine IL-4 production was compared in supernatants of Th2-skewed splenocyte culture. The *L. paracasei* strain KW3110 was a potent inducer, while the *L. delbrueckii* strain B was a poor inducer of IL-12 (Fig. 1). Repression of IL-4 production was observed only with *L. paracasei* strain KW3110. Otherwise, because these 2 LAB strains had such different immunomodulating activities in the mouse model, they were appropriate for subsequent comparative studies of immunomodulating activities in humans.

### EFFECT OF LAB GIVEN IN YOGURT ON TH1/TH2 RATIOS

In the group given yogurt with *L. delbrueckii* strain B, the Th1/Th2 ratio was significantly decreased (*P*=0.0052) after cedar pollen season as compared to the values of samples taken before the season began (Fig. 2). No significant changes were detected between the samples taken before the beginning and af-

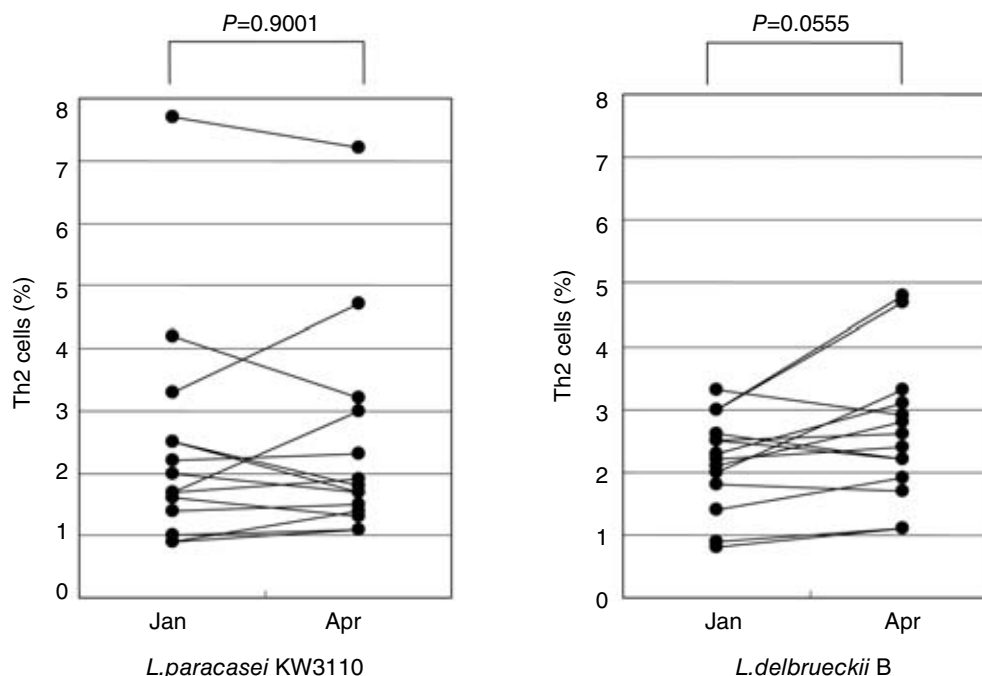


**Fig. 4** Percentages of Th2 cells before (Jan) and after (Apr) supplementation for 12 weeks with yogurt manufactured by either *L. paracasei* strain KW3110 or *L. delbrueckii* strain B.

ter the end of cedar pollen season in the *L. paracasei* strain KW3110 study group. To investigate whether the Th1 or Th2 cell counts were responsible for the differences between the two study groups, the population cell counts were compared rather than the ratios. Th1 cells were significantly decreased for both groups during cedar pollen season (Fig. 3). However, a trend toward increases in Th2 cells was observed in participants given the *L. delbrueckii* strain B ( $P=0.0555$ ). However no significant changes in percentages of either Th1 or Th2 cells were observed in the *L. paracasei* strain KW3110 study group (Fig. 4).

#### EFFECT OF LAB GIVEN IN YOGURT ON SERUM ECP

In the group given *L. delbrueckii* strain B yogurt, serum ECP was significantly increased ( $P=0.0157$ ) when the before and after cedar pollen season values were compared (Fig. 5). There were no significant changes in the group given the *L. paracasei* strain KW3110. Eosinophil count was significantly increased ( $P<0.01$ ) in both groups (data not shown).

#### DISCUSSION

We have previously shown that a wide variety of IL-12-inducing and IL-4-repressing activities are dependent upon the strain, not the species, of LAB.<sup>1</sup> The molecular characteristics that distinguish potent from weak immune stimulating bacteria are under investigation. Very small differences, such as carbohydrate antigen structure, may impact on the potential for im-

mune stimulation. To begin to analyze the basis of strain differences, we chose to use the *L. paracasei* strain KW3110 as a potent immune stimulator and the *L. delbrueckii* strain B as a weak immune stimulator. Using yogurt made by those LAB strains, the effects on human pollen allergy were investigated. Most of the previous clinical studies used placebo controls with no LAB, rather than comparing individual LAB strains, as immune modulators.<sup>2-5,11-13</sup> Such studies can estimate the optimum immune stimulating ability of LAB, but cannot account for the differences between various LAB strains. We were concerned that the comparative studies of LAB strains in humans would give unclear results. However the strain differences were evident when we compared results in participants fed yogurt made by the *L. delbrueckii* strain B with the group given *L. paracasei* strain KW3110 yogurt. The differences in Th2 cell counts are probably the reason for the differences between the two groups in the Th1/Th2 ratios. We have already shown that oral administration of KW3110 increased IL-12 secretion, decreased IL-4 secretion from splenocytes, but had no effect on IFN- $\gamma$  secretion.<sup>1</sup> In the present study, Th1 cell counts were significantly decreased in both study groups during the period of observation. Anti-allergic effects resulting from oral administration of LAB might be attributable to improvement of Th2 differentiation. The cedar pollen-specific IgE levels were significantly increased in both groups both before and after cedar pollen season. However, when the data from February and

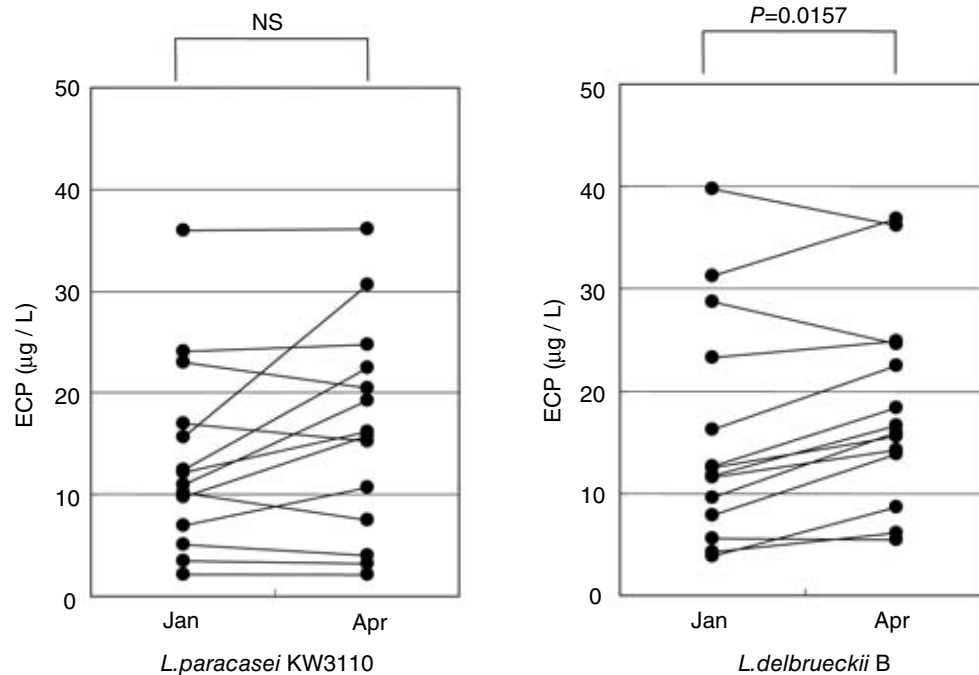


**Fig. 5** ECP values in study participants before (Jan) and after (Apr) supplementation for 12 weeks with yogurt manufactured by either *L. paracasei* strain KW3110 or *L. delbrueckii* strain B.

March were compared, there was a significant increase for the *L. delbrueckii* strain B but not for the *L. paracasei* strain KW3110 group (data not shown).

The amount of LAB ingested by test subjects was much lower than quantities of bacterial flora normally resident in the intestinal tract. Nonetheless, differences in immunological parameters were still observed. There may be gut immunity related recognition mechanisms that differentiate between foreign and inherent bacteria. The gut immune system may become tolerant to the inherent bacteria. The ingested LAB may enter the gut mucosa through the M cells that are located in Peyer's patches. Dendritic cells may also mediate bacterial uptake in the mucosal tissues.<sup>14</sup> The understanding of how bacteria enter the intestinal mucosa may help to explain the differences in anti-allergic effects between LAB strains.

*Lactobacillus rhamnosus* GG is one of the most extensively studied probiotic strains. Oral probiotic therapy with *L. rhamnosus* GG has shown promise as a therapeutic agent in small children with food allergy.<sup>15</sup> Also, *L. rhamnosus* GG is effective in preventing early atopic disease in children.<sup>5</sup> However oral treatment of *L. rhamnosus* GG had no effect on birch pollen allergy.<sup>16</sup> Our previous studies have shown that IL-12-inducing and IL-4-repressing activities of *L. rhamnosus* GG are only 50% of the activities of the *L. paracasei* strain KW3110.<sup>1</sup> The use of a potent immune stimulating strain of LAB for clinical trials of anti-allergic effects may be necessary to give consis-

tent results.

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