Lactic acid bacteria (LAB) might switch the Th2 biased immune response in allergic patients towards a balanced Th1/Th2 immune profile, leading to amelioration of allergy. To select strains of LAB that could be of potential application for foods in controlling allergy, 35 bacterial strains were screened in vitro using murine splenocytes and peritoneal exudate cells (PECs). *Streptococcus thermophilus* AHU1838 (FERM AP-21009), and *Lactobacillus paracasei* subsp. *casei* AHU1839 (FERM AP-21010) enhanced the secretion of Th1 cytokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12). The two strains of LAB also up-regulated the expression of CD40, and CD86 in dendritic cells (DCs), and activated cytotoxic T lymphocytes (CTL). These two strains could therefore be used in producing fermented food products that can enhance the Th1 immune profile which is important in ameliorating allergy.

**Key words:** lactic acid bacteria; Th1/Th2 immunobalance; allergy

Recent studies have demonstrated a significant increase in the prevalence of allergic diseases in industrialized countries. For instance, in the United States, it has been estimated that the percentage of allergic sufferers increased from 1–2% in the mid-1990s to 3.5–4% in 2005.1,2) Food allergies involve abnormal immunological responses to substances in foods regarded as harmless to healthy individuals and can be further subdivided into immunoglobulin E (IgE) and cell-mediated hyper-sensitivity.1,3) One theory of immunoregulation involves homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) activity.4) Cytokines produced by immunocompetent cells such as antigen-presenting cells (APCs) and T lymphocytes play a significant role in the modulation of allergy. There is a significant positive relationship between eosinophilic infiltration, nasal obstruction, air flow and Th2-derived cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 in patients with seasonal allergic rhinitis.5) Furthermore, IL-4 promotes the B lymphocyte production of IgE.6) In contrast, Th1 lymphocytes secrete interferon (IFN)-γ which is responsible for skewing a predominantly Th2 immune profile towards a Th1 profile and acts on B cells to inhibit switching to IgE.6) IFN-γ promotes the differentiation of naïve Th lymphocytes (Th0) towards a Th1 subset and prevents the proliferation of Th2 lymphocytes. In addition, myeloid DC subsets have a capacity to produce IL-12 in response to microbial stimuli and, thereby, to induce Th1 development.7)

It has been suggested that lactic acid bacteria (LAB) might switch the established Th2 immune response in allergic patients toward a long-lasting Th1 immune response and might therefore represent a new therapeutic strategy for the treatment of allergic diseases.8) LAB make contact with the immune system associated with the digestive tract and compete with allergens for the binding sites on the mucosal surface. Therefore, LAB might be used as a probiotic to prevent allergic diseases.9)
with the intestinal mucosa through M cells or follicle-associated epithelial cells from Peyer’s patches or through the epithelial cells of small or large intestine and induce the activation of signals associated with cytokine release. Moreover, it has been proposed that DCs localized in the gut-associated lymphoid tissue can sample bacteria directly from the intestinal lumen and modify the immune homeostasis. Clinical studies conducted in humans have indicated that LAB can alleviate the symptoms of atopy and perennial allergic rhinitis and enhance the anti-carcinogenic activity of the immune system. Since the bacterial properties required for the different applications are clearly distinct, selection of the LAB strain is very important for a more rational use of these organisms. Studies imply that not all immunoregulatory LAB strains are likely to be effective in promoting Th1-type anti-allergy immune responses. An increased understanding of the effects of clinically active probiotic bacteria on the immune system may enable the refinement of probiotic treatments for use under defined disease circumstances and enhance our knowledge of immune homeostasis in the specialized gut environment. Consequently, it has been suggested that an important criterion for the future selection of potential anti-allergy LAB strains should be their ability to induce the production of pro-Th1 molecules. The aim of this study was therefore to screen for potential strains of LAB that can induce a Th1 immune profile.

### Materials and Methods

#### Bacterial strains

The bacterial strains used in this study were obtained from Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan), National Institute of Technology and Evaluation, Biological Resource Centre (NBRC; Chiba, Japan), Japan Dairy Technical Association (Tokyo, Japan), and dairy processing plants in Japan, except for *Lactobacillus paracasei* HKW3110 which was isolated from a commercial product. All strains used in this study are listed in Table 1. *Lactobacillus paracasei* HKW3110 was used as a reference strain in all experiments, because it has been reported to have the potential for skewing the immune system towards a Th1 profile and for alleviating the symptoms of allergy disease. All the bacterial cultures were cultured under anaerobic conditions consisting of N₂, CO₂, and H₂ in the ratio of 8:1:1 at 37°C until the stationary growth phase. The strains of *Lactobacillus acidophilus, Lactobacillus casei* subsp. *casei*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus paracasei* subsp. *casei*, *Lactobacillus acidophilus* *Lactobacillus casei* subsp. *casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus paracasei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum* were cultured in de Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson Co., Sparks, MD, USA). The strains of *Streptococcus thermophilus* were cultured in M17 broth (Becton Dickinson Co.). Meanwhile the strains of *Bifidobacterium breve* and *Ruminococcus productus* were cultured in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan). The cultures were harvested by centrifugation at 3000 x g for 10 min at 4°C, and were then washed three times with sterile cold phosphate-buffered saline (PBS) and twice with sterile distilled water, heated killed at 105°C for 5 min, lyophilized, and suspended in PBS to a concentration of 10 mg/ml. LAB to be co-cultured with immunocompetent cells derived from mice was diluted with a RPMI-1640 (10% FCS) medium to the desired concentration. Unless otherwise stated, in all experiments a RPMI-1640 (10% FCS) medium not containing any bacterial strain was used as a control sample. The RPMI-1640 (10% FCS) medium was from the same stock as that used for diluting the bacterial samples.

#### Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories, (Yokohama, Japan). All of the mice used in this study were females of age 5–8 weeks and were maintained under the specific pathogen-free (SPF) conditions of Laboratory of Animal Experiment for Disease Model, Institute for Genetic Medicine, Hokkaido University.

#### Preparation of splenocytes

For *in vitro* studies, a RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum was used as a culture medium. The cultures were harvested by centrifugation, washed three times with saline, and were then washed three times with sterile cold phosphate-buffered saline (PBS) and twice with sterile distilled water, heated killed at 105°C for 5 min, lyophilized, and suspended in PBS to a concentration of 10 mg/ml. LAB to be co-cultured with immunocompetent cells derived from mice was diluted with a RPMI-1640 (10% FCS) medium to the desired concentration. Unless otherwise stated, in all experiments a RPMI-1640 (10% FCS) medium not containing any bacterial strain was used as a control sample. The RPMI-1640 (10% FCS) medium was from the same stock as that used for diluting the bacterial samples.

### Table 1. Bacterial Strains Tested in This Study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium breve</em></td>
<td>JCM1192, JCM7017, IKR1 (Ikaruga Milk Co.)</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>CSLA65, CSLA73, JCM1028, JCM1034</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em> subsp. <em>bulgaricus</em></td>
<td>JCM1001, JCM1002, JCM11039, NBRC13953, OH2 (Okayo Milk Co.), SB10164 55-1, SB10164 20-1</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> subsp. <em>paracasei</em></td>
<td>JCM1173</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> subsp. <em>casei</em></td>
<td>JCM1025, JCM5813, JCM8787, JCM8790, JCM8788</td>
</tr>
<tr>
<td><em>Lactobacillus kefiri</em></td>
<td>JCM5818</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> subsp. <em>casei</em></td>
<td>027 (Taiwan Technical Dairy Association)</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> subsp. <em>casei</em></td>
<td>AHI1839</td>
</tr>
<tr>
<td><em>L. paracasei</em></td>
<td>HKW3110 (Isolated from a yoghurt product)</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> subsp. <em>paracasei</em></td>
<td>OH3</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td>JCM1173</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>A4NZ12.4, AIP12.4, AZH12.4</td>
</tr>
<tr>
<td><em>Ruminococcus productus</em></td>
<td>OH1760</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>21072 (Hokkaido Food Processing Center, Sapporo, Japan), NBRC13957, AHU1838, OJ101, OJT102</td>
</tr>
</tbody>
</table>
LAB stimulation of splenocytes. Splenocytes obtained from C57BL/6 mice were cultured with LAB at final concentrations of 0.625, 1.25, 2.5, and 5 μg/ml in 96-well micro-titer plates. The final volume of the culture was 250 μl with a cell density of 2 × 10^6 cells/ml. After 2 d of culture, each supernatant was collected for IFN-γ and IL-4 quantification. A [¹³H]-thymidine uptake assay was used for checking the proliferation of LAB stimulated splenocytes and was performed in duplicate wells.

LAB stimulation of peritoneal exudate cells. C57BL/6 mice were injected intraperitoneally with 2 ml of a 10% thioglycolate medium. After 4 d, the mice were sacrificed, and peritoneal exudate cells (PECs) were obtained by washing the peritoneal cavity with ice-cold PBS. PECs were cultured in the RPMI-1640 (10% FCS) medium on plastic plates for 2 h at 37°C in a 5% CO₂ humidified atmosphere. The final volume of the culture was 250 μl with a cell density of 2 × 10^6 cells/ml. After 2 d of culture, each supernatant was collected for IL-10 and IL-12p70 quantification.

Two-step stimulation of splenocytes. In the first step, splenocytes obtained from BALB/c mice were cultured with LAB at 1 μg/ml at 37°C, in a 5% CO₂ humidified atmosphere for 36 h in a 24-well micro-titer plate. The final volume of the culture was 1 ml with a cell density of 2 × 10^6 cells/ml. The cultivated cells were collected and washed three times in RPMI-1640 (10% FCS) to remove the LAB cells.

In the second step, the splenocytes were cultured in the presence of 0.5 μg/ml of anti-mouse CD3 antibody at 37°C, in a 5% CO₂ humidified atmosphere for 36 h in 96-well micro-titer plate. After this culture, each supernatant was collected for IFN-γ and IL-4 quantification.

LAB stimulation of dendritic cells. Bone marrow cells obtained from BALB/c mouse femora were cultured in the presence of GM-CSF (30 ng/ml) and IL-3 (30 ng/ml), with or without LAB. L. paracasei HKW3110, S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 were co-cultured with bone marrow cells at concentrations of 5, 10, and 20 μg/ml in a 12-well plate (Costar, New York, NY, USA). The final volume of the culture was 1 ml with a cell density of 5 × 10^6 cells/ml. After 2.5 d of culture, the non-adhering lymphoid cells contaminating the bone cells were removed from the culture, and fresh medium containing cytokine cocktails and LAB was added. DCs harvested after 5 d of culture of the BM cells were used as bone marrow-derived dendritic cells (BMDC).

Measurement of cytokines. The concentrations of IFN-γ, IL-4, IL-10, and IL-12p70 in the cell culture supernatant was measured using a murine OptEIA ELISA set (BD Biosciences Pharmingen, CA, USA).

Flow cytometry. The phenotypic characterization of BMDCs was carried out by flow cytometry, using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) and CellQuest software (BD Biosciences). The mouse antibodies (mAb) used in our experiments were PE-conjugated anti-CD11c mAb, FITC-conjugated anti-H-2d, anti-I-A^d, anti-CD80, anti-CD86, and anti-CD40, purchased from PharMingen (San Diego, CA, USA). Anti-LFA (leukocyte function associated antigen)-1 mAb was purified from the supernatant of a hybridoma (KBA) and labeled with FITC-1 (Dojindo Laboratories, Kumamoto, Japan). Detailed procedures for staining were as described previously.26)

Cytotoxicity assay. The cytotoxicity of CTL generated by the allogeneic mixed lymphocyte reaction was measured by a 4 h ⁵¹Cr-release assay. H-2d-specific cytotoxicity was determined using P815 mastocytoma cells as targets. As a control, syngeneic MBL-2 T lymphoma cells (H-2b) were used. The percentage cytotoxicity was calculated as previously described.27,28)

Data analysis. Each experiment was independently replicated three times. Unless otherwise stated, the data obtained from the experiments were analyzed by one-way ANOVA using statistical computer software (Cohort 6.0, Monterey, CA, USA).

Results

Production of IFN-γ from splenocytes by LAB stimulation

Most of the LAB strains used in this study induced a high level of IFN-γ production by murine splenocytes derived from C57BL/6 mice (Fig. 1). The IFN-γ concentration in each culture supernatant depended on the LAB concentration added, and the optimal LAB
concentration was different for every strain. The highest concentration of IFN-γ was induced by *S. thermophilus* AHU1838 which was three times that of *L. paracasei* HKW3110 (the reference strain). On the other hand, *L. gasseri* JCM8787 did not induce IFN-γ production from murine splenocytes at all. After 2 d of culture, the proliferation of splenocytes stimulated with LAB at different concentrations was checked by a [3H]-thymidine uptake assay. The splenocytes proliferated in all cultures containing LAB (data not shown), indicating that the LAB concentrations used in this study were not toxic to murine splenocytes. This implies that the disparity in IFN-γ concentration was not due to any failure of the splenocytes to proliferate in some cultures, but rather was due to the difference in stimulatory ability of the different bacterial strains. In this experiment, the concentration of IL-4 in each culture supernatant was also checked, but no IL-4 was detected (data not shown). On the basis of the IFN-γ production, 15 strains of LAB that were able to induce a high level of IFN-γ concentration were selected for further evaluation. *L. gasseri* JCM8787 was included as a LAB strain with almost no immuno-stimulatory potential for comparative purposes.

**Production of IL-12p70 and IL-10 from PECs by LAB stimulation**

An antagonistic relationship was observed in the induction of IL-10 and IL-12 by some of the LAB strains (Fig. 2A and B). The concentrations of IL-12p70 and IL-10 produced after stimulation with LAB depended on
Fig. 2. Production of IL-10 and IL-12p70 by Peritoneal Exudates Cells (PEC) Co-Cultured with Selected LAB Strains at Concentrations of 1, 10, 100, and 1000 µg of LAB/ml.
the LAB concentration applied in the co-culture. Furthermore, the highest concentration of IL-12p70 produced after co-culturing with LAB and PECs varied according to the bacterial strain. The highest concentration of IL-10 produced by all bacterial strains was when LAB was added at concentration of 1000 μg/ml. The control induced neither IL-12p70 nor IL-10 production from PECs at all. The reference strain, L. paracasei HKW3110, induced moderate amounts of both IL-12p70 and IL-10. S. thermophilus 21072, L. delbrueckii subsp. bulgaricus NBRC13953, and L. delbrueckii subsp. bulgaricus JCM1002 induced a high level of IL-10 but a low level of IL-12p70. On the contrary S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 induced a high concentration of IL-12p70, but low levels of IL-10. The amounts of IL-12 induced by S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 were three to four times higher than by L. paracasei HKW3110. These results demonstrate that S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 could induce the production of IL-12 which is vital for the induction of a Th1 immune profile. We therefore selected five strains of LAB based on the IL-12p70 and IL-10 production characteristics for further trials.

Comparison of IFN-γ and IL-4 production from BALB/c T-cells by 2 step stimulation

The concentrations of IFN-γ and IL-4 in cultures of BALB/c splenocytes after 2-step stimulation were quantified. The cytokine level is expressed as a percentage of the control (100%) as shown in Fig. 3. Most LAB strains induced the secretion of a high concentration of IFN-γ, except for L. gasseri JCM8787 which was taken as a LAB strain with weak immunostimulatory potential. The statistical analysis results revealed a wide variation in IL-4 concentration, in comparison with the IFN-γ concentration induced by the various strains of LAB. S. thermophilus AHU1838 induced higher IFN-γ secretion in murine splenocytes than L. paracasei HKW3110. Stimulation of T-cells by anti-CD3 in the second step enhanced the production of cytokines, making it possible to clearly distinguish which LAB strains induced T-cells towards either the Th1 or Th2 phenotype.

Expression of co-stimulatory molecules after LAB stimulation

S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 were selected from the 35 bacterial strains tested on the basis of the results obtained by co-culturing PECs and the 2-step stimulation of splenocytes. In order to determine the maturation patterns of DCs exposed to different LAB strains, the expression of H-2d (MHC class I), I-Aδ (MHC class II), CD40, CD80 (B7.1), CD86 (B7.2), and LFA-1 (leukocyte function-associated antigen-1) on BMDCs was analyzed by flow cytometry. Histograms for the expression of co-stimulatory molecules on BMDCs are shown in Fig. 4. There was no change in the expression of H-2d, I-Aδ and LFA-1 after co-incubating BMDCs with LAB (data...
not shown). Meanwhile, the expression of CD40 and CD86 was significantly enhanced \((P < 0.05)\) in BMDCs stimulated with *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 when compared to unstimulated BMDCs. In respect of CD40, the mean fluorescent intensity was 118.91, 176.95 and 159.58 for unstimulated BMDCs (control), BMDCs stimulated with *S. thermophilus* AHU1838 and BMDCs stimulated with *L. paracasei* HKW3110 respectively. Meanwhile, for CD86, the mean fluorescent intensity was 126.99, 277.51, and 205.06 for unstimulated BMDCs (control), BMDCs stimulated with *S. thermophilus* AHU1838 and BMDCs stimulated with *L. paracasei* HKW3110 respectively. Most notably, the expression of CD40 was up-regulated by *S. thermophilus* AHU1838, but not by *L. paracasei* subsp. *casei* AHU1839. This up-regulation of co-stimulatory molecules, particularly by *S. thermophilus* AHU1838, indicates its ability to induce DC maturation.

### Cytotoxicity assay after the allogenic mixed lymphocyte reaction

C57BL/6(H-2b) derived murine splenocytes were stimulated with Mitomycin C (MMC)-treated BMDC derived from BALB/c(H-2b) for 4 d. After MLR, the cytotoxicity of CTL that had been generated was measured by a 4 h \(^{51}Cr\)-release assay. Unstimulated BMDCs exhibited very weak cytotoxicity, meanwhile BMDCs stimulated with *S. thermophilus* AHU1838 induced strong cytotoxicity at every concentration tested in this experiment. Furthermore, *S. thermophilus* AHU1838 induced higher cytotoxicity than *L. paracasei* subsp. *casei* AHU1839. There was no significant \((P < 0.05)\) difference in the CTL activity between splenocytes stimulated by *L. paracasei* HKW3110 and unstimulated splenocytes. The splenocytes stimulated by *L. paracasei* subsp. *casei* AHU1839 showed the highest CTL activity when induced at a concentration of 5 μg of LAB/ml whereas, with *S. thermophilus* AHU1838, it was 20 μg of LAB/ml. This indicates that the optimum concentration of LAB needed for the activation of CTL depended on the strain of LAB. These results show that *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 could significantly enhance the CTL activity associated with an elevated Th1 immune response.

### Discussion

Allergy is caused when the Th1/Th2 profile is biased towards a Th2 profile and it has been reported that LAB might modulate the allergic conditions that result from Th2 skewing amplification of the Th1 response, resulting in normalization of the Th1/Th2 balance.\(^4,16\) In this study, 35 bacterial strains were examined for their immunomodulatory effects; specifically for the ability to stimulate Th1 cytokines secretion in *in vitro* models. Our results show that *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 enhanced IL-12 secretion by PEC. Since DCs are crucial for the induction of antigen-specific immune responses such
as oral tolerance and Th1/Th2 immunity, the presence of probiotic bacteria or their components during the development of DC play a role in determining the outcome of the response. In human leukocyte cultures, development of DC play a role in determining the presence of probiotic bacteria or their components during the oral tolerance and Th1/Th2 immunity, the presence of probiotic bacteria or their components during the development of DC play a role in determining the outcome of the response.29) In human leukocyte cultures, the key source of IL-12 has been suggested to be DCs.30) It has been demonstrated that an enhanced capacity to express crucial co-stimulatory molecules and IL-12 by DCs was important for the differentiation and activation of T cells along the Th1 pathway.22,30) Moreover, IL-12 production by myeloid DCs induced by lactobacilli was not reversible when the myeloid DCs were simultaneously treated with E. coli lipopolysaccharide.23) Therefore, S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 could have the potential to skew the immune system towards a Th1 profile.

We evaluated the immuno-stimulatory effect of LAB by determining the amount of IFN-γ, which is a Th1 type of cytokine, produced by murine spleen cells co-cultured with LAB at various concentrations. The amount of IFN-γ produced by splenocytes stimulated with LAB differed according to the strain and concentration. For instance, L. gasseri JCM8787 and L. delbrueckii subsp. delbrueckii NBRC3202 showed wide disparity in stimulation of IFN-γ production by splenocytes. Meanwhile B. breve IKR1 showed a wide variation in the concentration affecting IFN-γ production. These results show that a small difference in the concentration of LAB resulted in a wide disparity in IFN-γ production by murine splenocytes. Consequently, it is not reliable to define the effect of LAB on IFN-γ production by murine splenocytes based on one concentration. In preliminary trials, we determined the optimal LAB concentration to be within the range of 1 mg/ml to 1 ng/ml, and the optimum time for co-culturing of splenocytes with LAB was 48 h. Screening strains of LAB with immuno-stimulatory properties can be easily evaluated by comparing the amount of cytokines produced when LAB is co-cultured with animal cells. However, there is a possibility that some potential LAB strains might be overlooked. Consequently, immuno stimulatory effect of LAB should be determined at various concentrations. On this basis, 13 strains of LAB were selected and two more strains were included, L. gasseri 8787 as a LAB strain with very weak immuno-stimulatory ability and L. paracasei HKW3110 as the reference strain. Therefore, a total of 15 strains of LAB were selected for further evaluation.

In some studies, only IL-12, which is a Th1 cytokine produced after a co-culture of LAB and PECs, was checked when screening for LAB. However, we also checked IL-10 to compare the amount of IL-10 which is anti-inflammatory and is a T-cell regulatory cytokine. The amounts of IL-12 and IL-10 differed depending on the strain of LAB. There was a high variation among the different strains of LAB in the induction of IL-12 and IL-10 by PECs. Considering a single LAB strain, we found that the optimal concentration for IL-12 and IL-10 production by PECs were different. The amount of IL-10 produced depended on the concentration of LAB. Some strains induced the production of high levels of both IL-12 and IL-10. For example, S. thermophilus OJT102 induced the production of high levels of both IL-12 and IL-10. On the contrary, L. gasseri JCM8787, which was used as a negative control, did not induce any cytokine production at all. L. delbrueckii subsp. bulgaricus JCM1002 and S. thermophilus 21072 induced the production of a high level of IL-10, but a low level of IL-12 whereas S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 induced a high level of IL-12 and low level of IL-10.

Recent reports indicate that reduced immune suppression rather than imbalance in the Th1/Th2 cell population is the key factor leading to the development of allergic diseases.31–33) It has been proposed that all types of microbial stimulation (both Th1 and Th2 polarizing) induce Tregs that control immune responsiveness through the production of immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β).34) In this study, we focused mainly on the ability of the selected strains of LAB to induce a Th1 immune profile. However, strains of LAB such as L. delbrueckii subsp. bulgaricus NBRC13953, L. delbrueckii subsp. bulgaricus JCM1002 and S. thermophilus 21072 with the potential to induce IL-10 might also suppress allergy. Furthermore IL-10 inducing strains of LAB could be of importance in modulating diseases associated with a Th1-biased immune profile such as inflammatory bowel disease (IBD). Therefore, there is a need to critically evaluate the immunomodulatory strains of LAB for developing tailor-made probiotics for specific disease groups. Beside their proinflammatory property, Th1 cells can act as regulatory effector cells and limit their own effector response in a negative feedback loop.35) However, the relationship between the self-limitation of Th1-mediated immunity by IFN-γ and Treg cells has not yet been thoroughly investigated. Elucidating the effects of different strains of LAB that can induce a Th1 immune profile on Treg cell development and functions will aid in establishing the safety of LAB and the regulation of Th1 immune responses.

The reason why the immuno-stimulatory ability of LAB varies according to strain has not yet been fully clarified. However, it has been reported that the observed strain-dependent stimulatory ability for IL-12 (p70) production arose, at least in part, from the amount of peptidoglycan present in gram-positive bacteria and that an intact cell wall was essential for the induction of IL-12.36,37) Determination of the amounts of IL-12 and IL-10 produced by PECs co-cultured with LAB helps in correcting screening of strains which either induces IL-12 or IL-10 production. Based on this result, we selected five strains for further evaluation; two strains induced the production of a relatively high concentration of IL-12, and three strains induced the production of a
high concentration of IL-10 by PECs. *L. paracasei* HKW3110 and *L. gasseri* 8787 were included as a reference and weak immunomodulatory strains respectively.

One of the methods for evaluating Th1/Th2 immunobalance in the screening of LAB is to make a mouse model which is sensitive to OVA alburn by an intraperitoneal injection of OVA alburn to a BALB/c mouse. This mouse is then used for the evaluation of Th1/Th2 immunobalance which is essential for proper screening of LAB. Spleen cells obtained from the OVA-sensitive mouse are then co-cultured with LAB. The production of IL-12, which is a Th1 cytokine and of IL-4, which is a Th2 cytokine, by the splenocytes is then compared to identify the strain of LAB which produces a high amount of IL-12 and low amount of IL-4. However, this method is quite complicated; it takes at least 2 weeks to prepare model mouse and 1 more week to co-culture the derived splenocytes and LAB. We tried this method and there were some individual differences in the response of the mouse. It is difficult to obtain valid results using this method because it requires great skill and experience. Furthermore, OVA is a virtual antigen so the presence of OVA might not depict the actual environmental conditions. We developed a new method to evaluate the effect of LAB on Th1/Th2 immunobalance.

The developed method involves two-step stimulation of splenocytes as described in the Materials and Methods section. The reference strain, *L. paracasei* HKW3110, induced the production of a high concentration of IFN-γ and low concentration of IL-4 which is essential for skewing the immune system towards a Th1 profile. Unlike the negative control, *S. thermophilus* AHU1838 showed good results. It changed the characteristics of murine T cells by enhancing IFN-γ in response to the α-CD3 antibody which is a non-specific T-cell stimulator. There was no significant difference (P > 0.05) between the concentration of IL-4 induced by *S. thermophilus* AHU1838 and the control. From these results, *S. thermophilus* AHU1838 had the ability to modulate the Th2-biased profile associated with allergy towards a Th1 profile. Hence, the new two step stimulation method is important for evaluating the influence of LAB on Th1/Th2 immunobalance. Results can be obtained within 3 d and it is an easy and rapid method for studying the immunomodulatory effects of LAB. Our results show that *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 had very good stimulatory ability on PECs. Therefore, to evaluate this effect further, *S. thermophilus* AHU1838, *L. paracasei* subsp. *casei* AHU1839, and *L. paracasei* HKW3110 as a reference strain were used to determine the effect of LAB on the maturation of DCs. *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 induced the production of cytokines and enhanced the expression of CD40 and CD86 on DCs. The expression of CD40 and CD86 modulates the immune system towards a Th1 profile. Furthermore, unmethylated CpG dinucleotides in the DNA of bacteria may suppress allergic response by directly suppressing IL-4/CD40-induced, Th2-related IgE and IgG1 production of B cells by inhibiting isotype switching from IgM to IgE and IgG1.38 CD40L appears to be the most if not the only effective stimulus, since it induced massive amounts of bioactive IL-12 in both primary and cultured DCs.39,40 Results obtained in this study show that *S. thermophilus* AHU1838 up-regulated the expression of both CD40 and CD86 on the surface of BMDCs. This indicates that *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1838 not only enhanced the secretion of Th1 cytokines but also up-regulated the expression of DC surface molecules which are important in the polarisation of the immune system towards a Th1 profile.

In this study, *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 activated the cytotoxicity of CTLs. However, the reference strain, *L. paracasei* HKW3110, did not activate the cytotoxicity of CTLs. This might be attributable to its inability to induce the expression of cell surface molecules on DCs which is required for priming CTL. Yoghurt fermented by *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 was acceptable to consumers (data not shown). Most immunomodulatory strains of LAB are added to fermented milk products that have been produced using strains of LAB with unknown immunomodulatory properties. This could limit the effect of the immunomodulatory strain that has been added to the fermented product. For instance, great differences were observed between *Lactobacillus reuteri* and *Lactobacillus casei* in their capacity to induce not only the production of key cytokines such as IL-12 and IL-10, but also maturation of cell surface markers, especially B7-2 (CD86), indicating that these bacteria may differentially alter antigen presentation in the gut and thus differentially affect the steady-state level of DC activation.30 Similarly, the co-incubation of *L. salivarius* CECT5713 or *L. fermentum* CECT5716 with murine bone marrow-derived macrophages stimulated the production of cytokines, although the effect of each strain was different.41 We found that *S. thermophilus* 21072, *L. delbrueckii* subsp. *bulgaricus* NBRC13953 and *L. delbrueckii* subsp. *bulgaricus* JCM1002 enhanced IL-10 production by PEC. There is thus the possibility that the Th2-biased immunomodulatory effects of *S. thermophilus* 21072 and *L. delbrueckii* subsp. *bulgaricus* NBRC13953 might influence the Th1-biased profiles of strains of LAB such as *S. thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 when all these strains are included in the same product. It is important to consider the immunomodulatory properties of the starter cultures used in the preparation of fermented products to which strains of LAB that can suppress allergic responses are to be added. In conclusion, there is a possibility that regular consumption of
yoghurt fermented with S. thermophilus AHU1838 and L. paracasei subsp. casei AHU1839 could be used as a powerful remedy for restoring Th1/Th2 immunobalance, because the two strains might have synergistic immunomodulatory properties.

Acknowledgments

We thank the Japan Small Scale Dairy Processors Association for providing us with some of the bacterial strains and partially funding this study. We are also grateful to members of Prof. Dr. Takashi Nishimura’s laboratory for the technical support they provided during the course of this study.

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