

RESEARCH ARTICLE

Oral administration of live *Lactococcus lactis* C59 suppresses IgE antibody production in ovalbumin-sensitized mice via the regulation of interleukin-4 production

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Lactococcus lactis; IgE antibody; IL-4; probiotics.

Introduction

IgE-mediated allergic diseases, such as pollen allergy, food allergy, and atopic dermatitis, are becoming serious public health problems in many countries. They are associated with elevation of antigen-specific IgE antibodies due to inadequately restrained T-helper (Th) type 2 response. Oral administration of heat-killed *Lactobacillus* strains inhibited antigen-specific serum IgE secretion in ovalbumin (OVA)-sensitized mice (Fujiwara *et al.*, 2004; Sashihara *et al.*, 2006; Torii *et al.*, 2007; Nonaka *et al.*, 2008; Segawa *et al.*, 2008). Also, some studies reported that the oral administration of live strains exerted a suppressive effect on serum OVA-specific IgE levels in OVA-sensitized mice (Ishida *et al.*, 2003; Ohno *et al.*, 2005) and antiallergic effects in human (Matsumoto *et al.*, 2007; Tamura *et al.*, 2007). Most of these studies have suggested that these lactic acid bacteria (LAB)

Abstract

To explore the potential probiotic effects of dairy starter strains to suppress an IgE allergic response, 10 strains of live dairy lactic acid bacteria were screened for their ability to stimulate the T-helper (Th) type 1 response that counteracts the Th2 response. Four strains with distinct patterns of interleukin(IL)-12p70 and interferon- γ production by murine splenocytes were then orally administered to Balb/c mice, and serum IgE antibody production was examined after ovalbumin sensitization. Oral administration of live *Lactococcus lactis* strain C59 significantly reduced the total IgE antibody levels, whereas oral administration of the other three strains had no effect on the total IgE levels in ovalbumin-sensitized mice. This inhibitory effect on IgE antibody production was lost when heat-killed C59 was used for oral administration. *Ex vivo* experiments showed that IL-4 production upon stimulation with the anti-CD3 antibody was significantly reduced in splenocytes of mice with an oral administration of live strain C59 compared with the control. These results indicate that the inhibition of IgE antibody production in mice treated with live strain C59 was due to the suppression of IL-4 production.

strains exhibited an antiallergic effect through an improvement of the Th1/Th2 balance via induction of interleukin (IL)-12 production. On the other hand, some strains suppressed antigen-specific IgE production by inducing regulatory T (Treg) cells (Ohno *et al.*, 2005; Torii *et al.*, 2007; Nonaka *et al.*, 2008). Thus, LAB strains have great potential to treat allergic diseases.

Lactococci, which are used frequently as starter bacteria in the manufacture of cheese and fermented milk products, have been underestimated as probiotics because they have been assumed to be incapable of surviving in the gastrointestinal tract. However, our previous studies have shown that some lactococcal strains can tolerate a low pH and a high concentration of bile to survive in the gastrointestinal tract (Kimoto *et al.*, 1999, 2000, 2003). We have also investigated the ability of 46 different *Lactococcus lactis* strains to induce the production of cytokines IL-6, IL-12,

Table 1. Strains used in this study

Strain	Species	Culture collection (no.)	Origin
Bc-10	<i>Lactobacillus helveticus</i>	MAFF* (401102)	Edam cheese
L-52	<i>L. helveticus</i>	MAFF (401201)	Edam cheese
L-14	<i>Lactobacillus casei</i>	MAFF (401403)	Cheese
L-34	<i>Lactobacillus plantarum</i>	MAFF (401501)	Edam cheese
H-17	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	MAFF (400008)	Cheese starter
Hc-1	<i>L. lactis</i> ssp. <i>cremoris</i>	NIRD [†]	
1256	<i>L. lactis</i> ssp. <i>lactis</i>	Lab [‡]	Milk
772	<i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i>	Lab	Kefir
C59	<i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i>	Lab	Cheese [§]
C61	<i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i>	Lab	Cheese [§]

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[†]National Institute for Research in Dairying, UK.

[‡]Our laboratory collection.

[§]Strains C59 and C61 were isolated from the same cheese.

and tumor necrosis factor- α in a murine macrophage-like cell line, J774.1, and demonstrated strain-specific and live bacteria-specific immunomodulatory effects (Suzuki *et al.*, 2008). Furthermore, we found that the oral administration of *L. lactis* G50 suppressed the production of specific IgE antibodies against ovomucoid (OVM; a potential egg allergen) in OVM-sensitized mice and improved the Th1/Th2 balance (Kimoto *et al.*, 2004). These results suggest that *Lactococcus* strains have probiotic properties.

To explore the potential probiotic effects of dairy starter strains, we examined the ability of 10 strains of live LAB to stimulate the production of Th1 cytokines by murine splenocytes, and four strains with distinct cytokine production were selected for further evaluation *in vivo*. The effects of oral administration of live strains on IgE antibody production were examined in OVA-sensitized mice. The underlying mechanism was further studied using a live and heat-killed strain and using *ex vivo* experiments.

Materials and methods

Materials

RPMI 1640 medium, *Escherichia coli* lipopolysaccharide, and fetal calf serum were purchased from Sigma-Aldrich (St. Louis, MO). M17 broth and Lactobacilli MRS broth (MRS) were purchased from Difco (Becton Dickinson, Sparks, MD). Anti-CD3/TCR monoclonal antibody, biotinylated rat anti-mouse IgE, IgG1, and IgG2a antibodies, mouse Opt EIA kits for IL-12p70, IL-10, interferon (IFN)- γ , and IgE antibody, streptavidin–horseradish peroxidase (HRP) conjugate, peridinin–chlorophyll–protein complex (PerCP)-conjugated anti-CD4 antibody (Rat, IgG2a, RM4-5), fluorescein isothiocyanate [fluorescein isothiocyanate (FITC)]-conjugated CD44 antibody (Rat, IgG2b, IM7), and phycoerythrin-conjugated CD62L antibody (Rat, IgG2a,

MEL-14) were purchased from BD Biosciences (Pharmingen, San Diego, CA). FITC-conjugated glucocorticoid-induced tumor necrosis factor receptor (GITR) (Rat, IgG2b, YGITR765) was purchased from BioLegend (San Diego, CA). Allophycocyanin (APC)-conjugated CD25 antibody (Rat, IgG1, PC61.5) was purchased from eBioscience (San Diego, CA). OVA was prepared from the whites of fresh eggs from White Leghorn hens using the ammonium sulfate method (Marshall & Neuberger, 1972) and freeze-dried.

Bacterial strains and growth conditions

The *Lactococcus* and *Lactobacillus* strains used are summarized in Table 1. The *Lactococcus* strains were cultured for 18 h at 30 °C in M17 broth supplemented with 0.5% glucose (GM17) and the *Lactobacillus* strains were cultured in MRS for 18 h at 37 °C.

Bacterial cells were harvested by centrifugation at 4000 g for 15 min at 4 °C, and then washed twice with 0.85% saline. Washed cells were suspended in RPMI medium to 100 μ g wet weight mL⁻¹ for incubation with splenocytes or in 0.85% saline to 350 μ g dry weight mL⁻¹ for oral administration in mice. To obtain heat-killed cells, the cell suspension was heated at 100 °C for 50 min.

Mice

Five-week-old female BALB/c mice were purchased from Charles River Japan Inc. (Kanagawa, Japan) and acclimatized for 1 week before the start of the experiment. Three or four mice were housed in a cage under a 12-h light/dark cycle and allowed free access to water. The basic feed was a nutritionally complete diet (MM-3, Funabashi Farm, Chiba, Japan). All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee, National Institute of Livestock and Grassland Science, Japan).

Oral administration of LAB and immunization

The schedules used in Experiments 1, 1', 2, 2', and 3 are shown in Fig. 1. Experiments 1 and 1' consisted of five groups (four LAB strains and saline, $n=10$ per group) and three groups (C59, H-17 and saline, $n=6$ per group), respectively. Experiments 2 ($n=8$ or 9 per group) and 2' ($n=6$ per group) consisted of three groups (live and heat-killed C59 and saline). Experiment 3 consisted of two groups [live C59 and phosphate-buffered saline (PBS), $n=8$ per group].

Mice in each group received 0.2 mL of a bacterial suspension containing one bacterial strain (70 µg dry weight) in 0.85% saline for 7 days, using an intragastric stainless-steel feeding tube. Control mice received 0.2 mL of 0.85% saline instead of the bacterial suspension. In Experiments 1, 1', and 2, OVA (100 µg) and alum precipitate (1 mg) as an adjuvant suspended in 100 µL of PBS were intraperitoneally injected on days 14 and 28 for sensitization. On day 35, blood samples were obtained from the tails. After 1 h of incubation at room temperature, the samples were centrifuged at 10 000 g for 95 s, and the sera obtained were stored at -80°C until antibody quantification. In Experiment 2', OVA and alum were injected on day 14. On day 21, mice were anesthetized with 75 µL of 0.05% pentobarbital and sacrificed by cervical dislocation. The splenocytes were prepared as described below. In Experiment 3, after 7 days of oral administration, mice were sacrificed and the splenocytes were prepared.

To test the survival of the strain C59 in feces, mice once received 0.2 mL of 0.85% saline ($n=3$) or a bacterial suspension containing 2×10^8 cells (200 µg dry weight) in 0.85% saline ($n=3$). Feces from each mouse were collected at 6, 24, and 48 h after oral administration, homogenized in 0.85% saline at a concentration of 0.1 g mL^{-1} , serially diluted, and spread on MRS plates. After 2 days of incubation at 30°C , formed colonies were used for colony hybridization. Two independent experiments were performed.

Cytokine production by splenocytes *in vitro*

Splenocytes were taken out from mice aseptically, teased apart, and passed through a cell strainer (BD Biosciences,

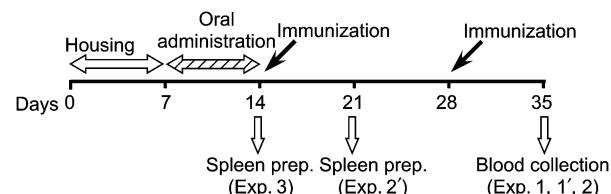


Fig. 1. Schedule of animal experiments. Oral administration of the test sample, immunization of mice with OVA and alum, and collection of the blood samples and preparation of spleens are shown.

Canaan, CT). After the removal of the erythrocytes using ACK buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA, pH 7.2), the cells were washed twice with RPMI 1640 medium and then resuspended in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (FCS), 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, and 50 μM 2-mercaptoethanol (modified RPMI).

The splenocytes from untreated mice were seeded into each well of 24-well culture plates (6×10^6 cells mL^{-1} per well). Bacterial cells of each LAB strain were added in the culture (10 µg wet weight mL^{-1}) and were maintained at 37°C in 5% CO_2 –95% air. After a 72-h incubation, culture supernatants were collected by centrifugation and frozen at -80°C until cytokine analysis.

The splenocytes from once OVA-sensitized control mice (Experiment 2') were seeded into each well of 96-well culture plates (8×10^5 cells/ 0.1 mL^{-1} per well). Fifty microliters of serially diluted bacterial suspension of each LAB strain (4 ng–400 µg mL^{-1}) and 50 µL of OVA (400 µg mL^{-1}) were added in the culture. After a 96-h incubation, culture supernatants were collected and cytokines were analyzed.

The splenocytes from once OVA-sensitized mice (Experiment 2') were cultured in 24-well culture plates (4×10^6 cells mL^{-1} per well) in the presence of 0, 1, 10, and 100 µg mL^{-1} of OVA. Culture supernatants were collected after 96 h for cytokine analysis.

The splenocytes from strain C59- or PBS-administered mice (Experiment 3) were cultured in 24-well culture plates after the plates (4×10^6 cells mL^{-1} per well) were coated with 10 µg mL^{-1} anti-CD3 antibodies for 2 h at 37°C . Culture supernatants were collected after 24 h and cytokines were analyzed. CD4^+ T cells were purified from splenocytes from strain C59- or PBS-administered mice (Experiment 3) using an anti-CD4 monoclonal antibody coupled to MACS microbeads (Myltenyi Biotec, Bergisch Gladbach, Germany) as specified by the manufacturer. Purified CD4^+ T cells (10^5 cells mL^{-1} per well) were seeded in 24-well culture plates after the plates were coated with 10 µg mL^{-1} anti-CD3 antibodies for 2 h at 37°C . Culture supernatants were collected after 96 h.

Flow cytometry

Single-cell suspensions prepared from spleens were stained and analyzed on Gallios (Beckman Coulter Inc., Fullerton, CA) using KALUZA software to determine the phenotype of splenocyte populations.

Cytokine and immunoglobulin analysis by enzyme-linked immunosorbent assay (ELISA)

Determination of IL-12p70, IL-10, IL-4, and IFN- γ levels in culture supernatants and the total IgE antibody levels in sera

was performed using sandwich ELISA (Mouse Opt EIA kits) as described previously (Kimoto *et al.*, 2004). OVA-specific IgE, IgG1, and IgG2a antibodies in sera were analyzed by ELISA with biotinylated rat anti-mouse IgE, IgG1, and IgG2a antibodies and streptavidin–HRP conjugate as described previously (Kimoto *et al.*, 2004).

Colony hybridization

Feces suspensions were serially diluted and spread on MRS plates. After 2 days at 30°C, colonies were transferred on a nylon membrane (Hybond N plus, Amersham). The C59-specific PCR fragment (manuscript in preparation) was amplified using the C59HF primer (5'-CACTTTCTAGAT TAGACATGAAGC-3') and the C59HR primer (5'-CGA TAAATACCCCTGACCTACACC-3'). The 800-bp product (DDBJ accession no. AB599929) was labeled using a Dioxigenin (DIG) High Prime Kit (Roche Diagnostics GmbH). Colony hybridization was performed using a DIG-labeled probe and an anti-DIG antibody alkaline phosphatase conjugate according to the manufacturer's instructions.

Statistical analysis

Statistical analysis using the general linear model (GLM) procedure with one-way allocation was performed using the SAS software (version 9.1, SAS Institute, Cary, NC). Comparisons of means were analyzed using Tukey's test for strain (Experiment 1) and treat (Experiments 2 and 3). The experimental data were expressed as least-squares means $\pm$ SE. Statistical differences of cytokine production by splenocytes between control mice and mice treated with live strain C59 were analyzed using an unpaired *t* test with Welch's correction using PRISM 4 (Graphpad Software Inc., La Jolla, CA). Differences were considered significant when the *P*-value for the effect was < 0.05 .

Results

Cytokine-stimulatory activities of LAB strains *in vitro*

To explore the potential probiotic effects of dairy LAB on IgE production, we first examined the ability of 10 dairy strains (Table 1) to stimulate the production of Th1 cytokines by murine splenocytes. IL-12p70 and IFN- γ are the key cytokines in Th1 immune responses, which can downregulate the Th2 response promoting IgE production. Whereas all of the live LAB strains examined here induced the production of IL-12p70 and IFN- γ by splenocytes, the level and pattern of these cytokine production were somewhat different among LAB species (Table 2). We selected four strains, H-17, L-14, Bc-10, and C59, which showed

Table 2. Effects of lactic acid bacteria on the production of IL-12p70 and IFN- γ by murine splenocytes

Strain*	Species	IL-12p70 (ng mL ⁻¹)†	IFN- γ (ng mL ⁻¹)†
H-17	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	7.94 $\pm$ 3.83	35.41 $\pm$ 14.66
L-14	<i>Lactobacillus casei</i>	7.53 $\pm$ 1.41	31.18 $\pm$ 1.70
772	<i>L. lactis</i> ssp. <i>lactis</i>	6.41 $\pm$ 3.49	33.23 $\pm$ 13.16
C61	<i>L. lactis</i> ssp. <i>lactis</i>	6.39 $\pm$ 1.23	39.33 $\pm$ 6.47
L-34	<i>Lactobacillus plantarum</i>	4.18 $\pm$ 1.54	21.94 $\pm$ 13.08
1256	<i>L. lactis</i> ssp. <i>lactis</i>	3.17 $\pm$ 0.03	42.56 $\pm$ 1.03
L-52	<i>Lactobacillus helveticus</i>	2.15 $\pm$ 0.53	32.80 $\pm$ 5.20
Hc-1	<i>L. lactis</i> ssp. <i>cremoris</i>	1.66 $\pm$ 0.79	21.86 $\pm$ 4.26
Bc-10	<i>L. helveticus</i>	1.26 $\pm$ 0.26	24.61 $\pm$ 22.08
C59	<i>L. lactis</i> ssp. <i>lactis</i>	0.00 $\pm$ 0.00	14.95 $\pm$ 12.22

*Strains are represented in rank order of IL-12p70 induction.

†Data are presented as means $\pm$ SE of two independent experiments.

distinct patterns of IL-12p70 and IFN- γ induction, for further evaluation of their regulatory capability of IgE production *in vivo*. Thus, strains L-14 and H-17 strongly induced IL-12p70 and IFN- γ production by splenocytes. Bc-10 induced IFN- γ production, but weakly induced IL-12p70. Strain C59 had the weakest ability to induce the production of IL-12p70 and of IFN- γ .

Effect of oral administration of LAB strains on antibody production in OVA-sensitized mice

As shown in Fig. 1 (Experiment 1), four live LAB strains were administered orally to mice for 7 days, and then the mice were sensitized by two times of OVA and alum precipitate at 14-day intervals. Total IgE and OVA-specific IgE antibodies in the serum 7 day after the second OVA-injection were examined (Fig. 2). Administration of C59, which had the lowest IL-12p70 and IFN- γ production, significantly reduced the total IgE antibody level compared with that of the control group without LAB administration ($P < 0.05$; Fig. 2a). No significant difference was observed in the OVA-specific IgE antibody levels (Fig. 2b) and OVA-specific serum IgG1 and IgG2a antibody production in response to any of the four strains (data not shown). When live lactococcal strains of C59 and H-17 were used for the same procedure described above (Fig. 2, Experiment 1'), the concentrations of total IgE antibody of control, C59-, and H-17-administered mice were 9.59, 3.25 and 9.72 ng mL⁻¹, respectively, indicating that the administration of live strain C59 significantly reduced the total IgE antibody level compared with that of the control group. No significant difference was observed in the OVA-specific IgE antibody levels and OVA-specific serum IgG1 and IgG2a antibody production in response to live strain C59.

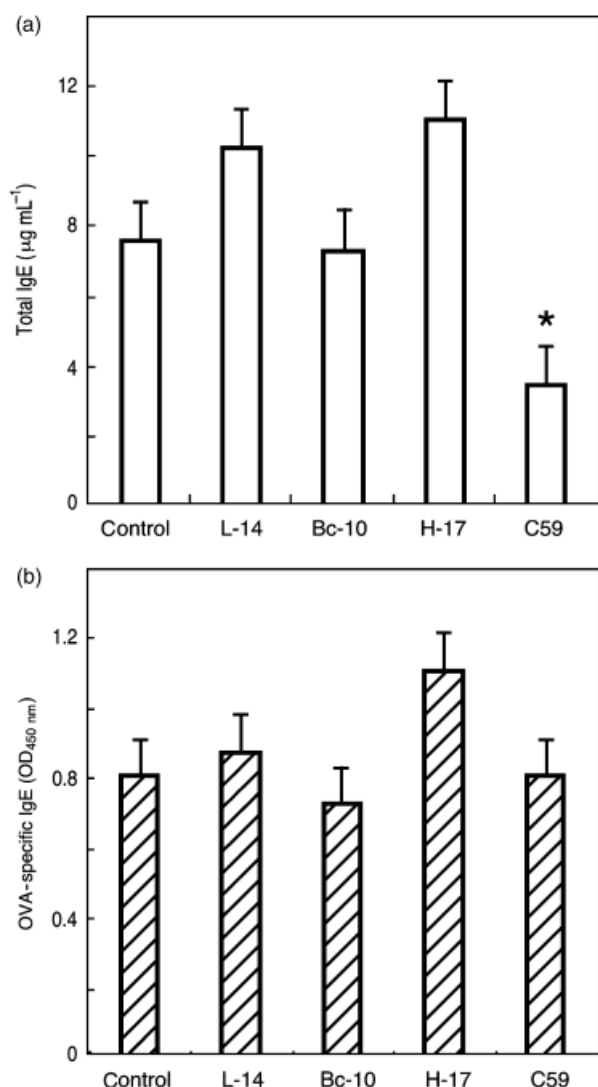


Fig. 2. Effect of oral administration of strain L-14, Bc-10, H-17, and C59 on the total and OVA-specific IgE antibody production in OVA-sensitized mice. (a) Total IgE antibody. Data are shown as means $\pm$ SE. Asterisk indicates a value significantly different from the control (* $P < 0.05$). (b) OVA-specific IgE antibody. Data are shown as means $\pm$ SE.

Effect of oral administration of live and heat-killed strain C59 on IgE antibody production in OVA-sensitized mice

To assess whether live bacterial cells are required for the suppressive effect of strain C59 on IgE antibody production, the total serum IgE antibody levels were compared in OVA-sensitized mice that had been pretreated with oral administration of heat-killed or live strain C59. The total IgE antibody production of live C59-administered mice was significantly ($P < 0.05$) reduced than that of control mice, whereas the total IgE antibody production was not significantly different between heat-killed C59- and live C59-

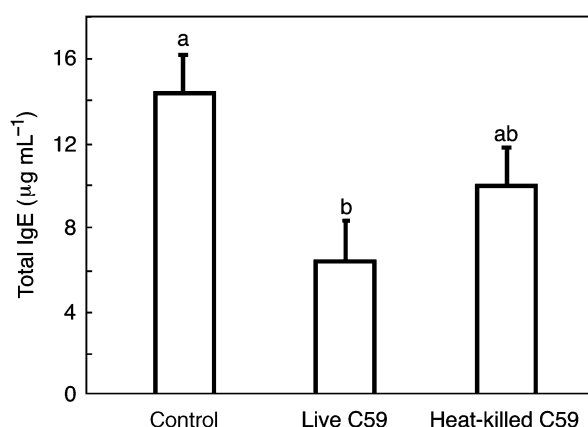


Fig. 3. Effect of oral administration of live or heat-killed strain C59 on the total IgE antibody production in OVA-sensitized mice. Data are shown as least-square means $\pm$ SE ($n = 8$ or 9) and different lower case letters (a, b) indicate statistical significance ($P < 0.05$). Data are shown from one set of experiments and are representative of two independent experiments.

administered mice (Fig. 3). The production of OVA-specific serum IgE, IgG1, and IgG2a antibodies did not differ among groups (data not shown).

Effect of oral administration of live C59 on cytokine production and cell population in the spleen

To clarify the mechanisms involved in the suppression of IgE antibody production in mice, we examined cytokine production by total and CD4^+ -enriched splenocytes of mice that were orally given PBS or live strain C59 (Fig. 1, Experiment 3). IL-4 production by total splenocytes in the presence of anti-CD3 antibodies was significantly ($P < 0.05$) decreased by oral administration of strain C59 after 24 h of incubation (Fig. 4a). Two independent experiments resulted in a significant decrease of IL-4 in the total splenocytes from C59-administered mice. Oral administration of heat-killed strain C59 ($70 \mu\text{g day}^{-1}$) and a 10-fold increase of live C59 ($700 \mu\text{g day}^{-1}$) also significantly decreased IL-4 production by total splenocytes compared with the control, whereas no significant difference was observed among live, heat-killed, and 10-fold groups (data not shown). In the case of CD4^+ cells, the IL-4 production in the presence of anti-CD3 antibodies was decreased ($P = 0.05$) by oral administration of strain C59 after 96 h of incubation (Fig. 4a). Administration of live strain C59 did not significantly alter the levels of production of IFN- γ (Fig. 4b), IL-10 (Fig. 4c), or TGF- β (data not shown) in splenocytes or CD4^+ cells.

Because the production of IL-4 was suppressed in splenocytes from C59-administered mice, we examined whether or not the frequency of CD4^+ T cells was increased by oral

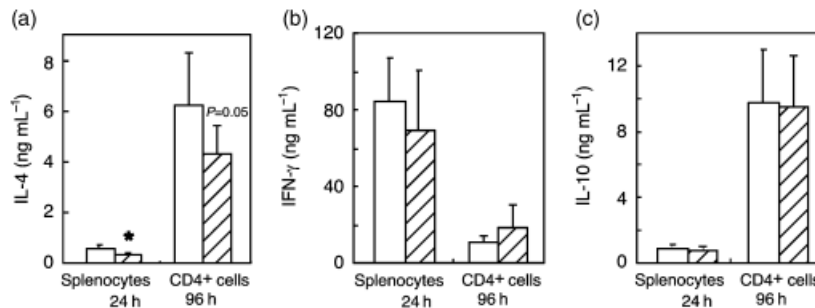


Fig. 4. Effect of feeding of live strain C59 on cytokine production by murine splenocytes stimulated with the anti-CD3 antibody. Production of IL-4 (a), IFN- γ (b), and IL-10 (c) by splenocytes is shown. BALB/c mice ($n = 7$) were fed a suspension of live strain C59 or PBS (control) for 7 days. The splenocytes were collected and stimulated on plates coated with the anti-CD3 monoclonal antibody ($10 \mu\text{g mL}^{-1}$). Open and hatched bars represent data from splenocytes from mice administered PBS and C59, respectively. Data are shown as means $\pm$ SD. Asterisk indicates values significantly different from the control (* $P < 0.05$). Data are shown from one set of experiments and are representative of two independent experiments.

administration of strain C59. Contrary to our expectation, there was no significant difference in the frequency of CD4⁺ cells in splenocytes between C59-administered mice and control mice ($27.9 \pm 3.97\%$ and $28.7 \pm 4.02\%$, respectively). The expression patterns of GITR⁺ and CD25⁺ in CD4⁺ cells were also examined because the expression of GITR has been reported to be a characteristic feature of CD4⁺CD25⁺ regulatory T cells (Shimizu *et al.*, 2002). However, no significant difference was observed in the frequency of GITR⁺CD25⁺ cells among CD4⁺ cells in splenocytes between C59-administered mice and control mice ($12.5 \pm 0.96\%$ and $12.0 \pm 1.21\%$, respectively). In addition, the frequency of CD62L^{low}CD44^{high}CD4⁺ cells with a memory phenotype in splenocytes was not significantly different between C59-administered mice and control mice (14.8 ± 1.73 and 13.8 ± 1.58 , respectively).

To examine the production of cytokines *ex vivo*, live and heat-killed strain C59 were administered and the splenocytes were restimulated with OVA (Fig. 1, Experiment 2'). The production of IL-4 from splenocytes of live or heat-killed C59-administered mice was always less than that of control mice in the presence of stimulation with OVA at any concentration (1 , 10 , and $100 \mu\text{g mL}^{-1}$), although the difference was not significant. No significant difference was observed in the production of IL-10, IFN- γ , and TGF- β from splenocytes between C59-administered mice and control mice.

Effect of LAB strains on the production of IL-4 and IFN- γ

To examine the effect of LAB strains on the production of IL-4 and IFN- γ from splenocytes, splenocytes from once OVA-sensitized control mice (Fig. 1, Experiment 2') were incubated with various concentrations of strains C59, H-17, and Bc-10 in the presence of $100 \mu\text{g mL}^{-1}$ of OVA. In the absence of LAB, splenocytes produced 350 pg mL^{-1} of IL-4

and 12.5 ng mL^{-1} of IFN- γ . The production of IL-4 decreased with increasing LAB concentration, whereas that of IFN- γ increased with increasing LAB concentration. Among three strains, H-17 was most effective in view of Th1/Th2 balance; the presence of $1 \mu\text{g mL}^{-1}$ of H-17 could completely suppress IL-4 production and increase IFN- γ production to 41 ng mL^{-1} . The production of IL-4 and IFN- γ in the presence of $10 \mu\text{g mL}^{-1}$ of strain Bc-10 was 8 pg mL^{-1} and 55 ng mL^{-1} , respectively, whereas that of strain C59 was 32 pg mL^{-1} and 32 ng mL^{-1} , respectively.

Survival of strain C59 in feces

Living cells of strain C59 ($2.6 \times 10^7 \text{ cells g}^{-1}$ feces) were detected in feces 6 h after oral administration by colony hybridization. No colonies from feces administered 0.85% saline were hybridized to the probe. No living cells of strain C59 were detected in feces 24 and 48 h after oral administration, suggesting that the strain C59 is delivered live to feces through the intestines, but cannot remain alive in the intestines.

Discussion

From a series of *in vitro* experiments with murine splenocytes, we selected four LAB strains with a distinct pattern of Th1 cytokine production (i.e. IL-12 and IFN- γ) for further evaluation of IgE antibody production in OVA-sensitized mice. Contrary to our expectation, oral administration of live strain *L. lactis* C59, which had the weakest ability to induce IL-12 and IFN- γ production, reduced the total IgE antibody levels. Suppression of total IgE production by oral administration of live strain C59 was observed in three experiments. However, oral administration of the other three strains, which had higher immunostimulatory activity *in vitro* than strain C59, had no effect on the total IgE levels (Fig. 2). Therefore, our results suggest that LAB strains with

superior *in vitro* Th1 cytokine induction activity do not always have the ability to suppress IgE antibody production.

Recent reports have demonstrated that lactobacilli that promoted Th1-type immune responses could suppress the production of both antigen-specific IgE and IgG1 serum antibody in mice (Murosaki *et al.*, 1998; Shida *et al.*, 1998, 2002). Similarly, oral administration of the lactococcal strain G50 suppressed the total and OVM-specific IgE serum levels in OVM-sensitized mice and increased IgG2a antibody production (Kimoto *et al.*, 2004). In contrast, our present results showed that oral administration of live C59 effectively suppressed the production of the total IgE antibody (Fig. 4), but did not affect OVA-specific serum IgE, IgG1, and IgG2a antibody levels in mice. Therefore, live strain C59 may suppress the production of total IgE antibody in a manner different from that of strain G50.

IL-4, which is produced by various kinds of cells including Th2 cells, promotes IgE class switching and augments IgE secretion from plasma cells (Geha *et al.*, 2003; Xu *et al.*, 2008; Kashiwada *et al.*, 2010). On the other hand, IFN- γ , a Th1 cytokine, inhibits IgE antibody production (Xu & Rothman, 1994; van Halteren *et al.*, 1997). In our results, IL-4 production in splenocytes and CD4⁺ cells from C59-administered mice upon *in vitro* stimulation with anti-CD3 antibody was decreased compared with that of control mice (Fig. 4a). On the other hand, IL-4 production in splenocytes from once OVA-sensitized mice was decreased, but the effect was not significant. Therefore, oral administration of strain C59 exhibited an immunoregulatory function to inhibit IL-4 production, which might result in the suppression of total IgE antibody production, but not antigen-specific IgE antibody production.

We have examined the gene expression profiles of ileum of C59-administered mice compared with control mice by microarray analysis. Using gene set enrichment analysis (GSEA), we discovered significant downregulation of *Locomotion_Behavior*, which include genes involved in the specific movement from place to place of an organism in response to external or internal stimuli (manuscript in preparation). The expressions of several genes encoding chemokines and chemokine receptors were significantly reduced in C59-administered mice compared with control mice, suggesting that the migration of immune cells in the ileum was rather downregulated in the presence of C59.

It has been shown that heat-killed LAB strains exhibited immunostimulatory properties (Murosaki *et al.*, 1998; Fujiwara *et al.*, 2004; Sashihara *et al.*, 2006; Torii *et al.*, 2007; Nonaka *et al.*, 2008; Segawa *et al.*, 2008). Whereas the heat-killing process should alter the cellular components of LAB, which are essential to the interaction with immune cells, there are few studies that have clarified the effect of live and heat-killed cells on IgE production. Our result showed that the oral administration of heat-killed strain C59 did not

reduce total IgE antibody production from OVA-sensitized mice (Fig. 3), suggesting that heat-sensitive molecules may be critical to the interaction between C59 and immune cells that are involved in the suppression of IgE production. By colony hybridization, we showed that C59 cells can pass through the gastrointestinal tract alive, but cannot proliferate in it. Therefore, it may be difficult for strain C59 to affect resident flora. Alternatively, C59 has capsular polysaccharide around the cell wall, which may be recognized by certain immune cells. Our preliminary *in vitro* experiment suggested that strain C59 evaded uptake by a macrophage-like cell line (data not shown), in which the existence of capsular polysaccharide may be involved. The mechanisms by which strain C59 interacts with the immune system in the alimentary tract remain to be elucidated.

In conclusion, we found that *L. lactis* strain C59 suppressed IgE production in OVA-sensitized mice when live cells were administered. Although strain C59, among ten LAB strains examined, had the lowest ability to induce IL-12 and IFN- γ production by murine splenocytes *in vitro*, oral administration of strain C59 suppressed IL-4 production by splenocytes. Our results suggest that *L. lactis* C59 may have the potential to improve cytokine balance for preventing IgE-dependent allergic diseases.

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