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REVIEW
Immunomodulatory Effects of *Lactococcus lactis* Strains

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Abstract

Strains of *Lactococcus lactis* are present in various natural environment or fermented products, and have been used as starters. In this review, we firstly discuss the immunomodulatory effects of *Lactococcus* strains from the perspective of their strain-specificity. Forty-six different *Lactococcus* strains were investigated for their ability to induce cytokine production in the murine macrophage-like cell line, J774.1. Strain-specificity and heat stability related to the ability of *Lactococcus* cells to induce interleukin (IL)-6, IL-12 and TNF-α, and induce cell necrosis or apoptosis were discussed. Secondly, to examine the immunomodulatory effects of *Lactococcus* cells in vivo, the effects of oral administration of four strains were investigated for their ability to regulate IgE production in ovalbumin-sensitized mice. Among the four strains, only *L. lactis* strain C59 showed a significant reduction in the total IgE antibody levels in serum. This suppressive effect on IgE antibody production was lost when strain C59 was heat-killed. Experiments using splenocytes of mice administered with live strain C59 indicated that the suppression of IgE antibody production by live strain C59 was due to the suppression of IL-4 production. The strain-specific immunomodulatory effects of *Lactococcus* strains are discussed.

Discipline: Food
Additional key words: apoptosis, IgE antibody, interleukin, lactic acid bacteria, macrophage

Introduction

Strains of the *Lactococcus* genus of lactic acid bacteria (LAB) are widely used as starter bacteria when manufacturing cheese and other fermented dairy products, and are also found in fermented vegetables. Because *L. lactis* is a bacterium "generally regarded as safe", it is the first species among LAB whose genome sequence has been determined[4] and is a candidate food-grade delivery vector of therapeutic proteins to intestinal mucosa[19]. In the NARO Institute of Livestock and Grassland Science (former National Institute of Livestock and Grassland Science; NILGS), researchers started isolating useful LAB strains for dairy products in the early 1950s, which were then kept as subcultures or freeze-dried stocks until the cryopreservation method had been established. These strains from the so-called old collection have been distributed and used as starter cultures in Japan. From 1999, NILGS has created a LAB library consisting of ca. 2600 strains isolated from about 200 samples such as fermented foods etc. This collection, together with the old collection, enabled us to screen strains beneficial for health.

In the late 1980s, the concept of probiotics was proposed as live microorganisms, conferring a health benefit to the host, and strains isolated from intestinal flora belonging lactobacilli. Strains of the genera *Lactobacillus* and *Bifidobacterium* are the most common probiotics used for human consumption. While *Lactococcus* strains have generally been assumed not to survive passage through the gastrointestinal tract, some strains have proved their ability to do so[12,14]. The potential usage of *Lactococcus* strains as probiotics was reviewed in JARQ by Kimoto-Nira et al.[13].

Recent metagenomic approaches have generated an entirely new perspective, suggesting that our microbiota might be involved in the development of obesity and related disorders such as metabolic syndrome[9,17,26]. Studies have demonstrated that these disorders may be associated with profound microbiotal changes. Thus, the potential of probiotics is expected to control the microflora in the gastrointestinal tract and improve lifestyle diseases.

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Microbial specific molecules known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs)[8] are recognized by surface and cytosolic receptors expressed in the gastrointestinal tract. For example, the toll-like receptor (TLR) 9 recognizes the CpG motif of bacterial DNA. A study using TLR9-deficient mice revealed that the anti-inflammatory effects of probiotics are mediated by their own DNA and that live microorganisms need not attenuate murine experimental colitis[21]. The oral administration of heat-killed LAB has been shown to be effective for modulating innate immune functions[22]. While probiotic effects may synergetically be caused by TLR-mediated immune responses, the process may be affected by the sensitivity of MAMPs to heat or enteric environment, such as gastric acid, bile and digestive enzymes. However, the molecular mechanism that endows health-promoting benefits to some specific strains remains unknown.

The purpose of this review is to introduce our research on the immunomodulatory effects of lactococci, which have been tested in vitro and in vivo, and emphasize strain-specificity. The difference in effects of live and heat-killed bacteria is also discussed.

Effects of *Lactococcus* strains on cytokine production of J774.1[23]

Nomura et al. selected *L. lactis* strains by *gadB*-targeted *L. lactis*-specific PCR from 2600 isolates of LAB, which were classified into 41 representative strains[18]. Using this collection and some dairy strains, a total of 46 strains of *Lactococcus* strains were investigated for their immunomodulatory activity to induce production of cytokines IL-12, IL-6, and TNF-α using the murine macrophage-like cell line, J774.1[23]. Fig. 1 shows the extent of the induction of IL-12p40 and TNF-α, which was strain-specific and unrelated to subspecies, biovars, or the sources of the isolates.

Among these strains, eleven strains with the distinct ability to induce cytokines were selected, and the effects of dose (low dose, OD_{620} 0.05; high dose, OD_{620} 0.25) and heat treatment (95°C for 10 min) of the strains on cytokine induction were investigated. To compare the effects of strains, the IL-12 induction ratio of high dose to low dose was calculated (Fig. 2). When live strains were added (gray bars), the ratios of strains O07, P17, 1175, J53 and 341 were very low, whereas those of strains P79 and S63 were high. When heat-killed strains were added (white bar), the ratios of all strains were kept high. These results suggest that a high dose of live cells of certain strains has an inhibitory effect on the cytokine production of J774.1 cells. Heat-killed cells induced more IL-6 production than live cells, as well as IL-12 production (data not shown), suggesting the stimuli required for IL-6 and IL-12 induction are heat-stable. Conversely, live cells of some strains, such as G50, S63, induced TNF-α production (Fig. 1), but the induction ability was lost after heat treatment (data not shown). Accordingly, the stimuli required for TNF-α induction are heat sensitive and differ from those required for IL-6 and IL-12 induction. The candidates of heat stable molecules that stimulate IL-6 and IL-12 production or MAMPs of *Lactococcus* strains may be the CpG motifs of bacterial DNA and/or peptidoglycan, which are the ligands of TLR9 and Nod2, respectively[24,25]. The candidates of heat sensitive MAMPs may be lipoproteins, which represent the ligand for TLR2[15], but cannot be used to explain strain-specific TNF-α induction. The amount or expression of strain-specific heat sensitive MAMPs should be investigated. Desirable strains for immunobiotics[7] are those that improve the immune function of hosts by increasing the number of IgA-producing plasma cells or increasing phagocytosis, as well as by increasing the proportion of Th1 and NK cells[20]. IL-12 is involved in the differentiation of naive T cells into Th1 cells and in the activation of NK cells[20]. Heat-killed *Lactococcus* cells could stimulate J774.1 cells to induce IL-6 and IL-12 but could

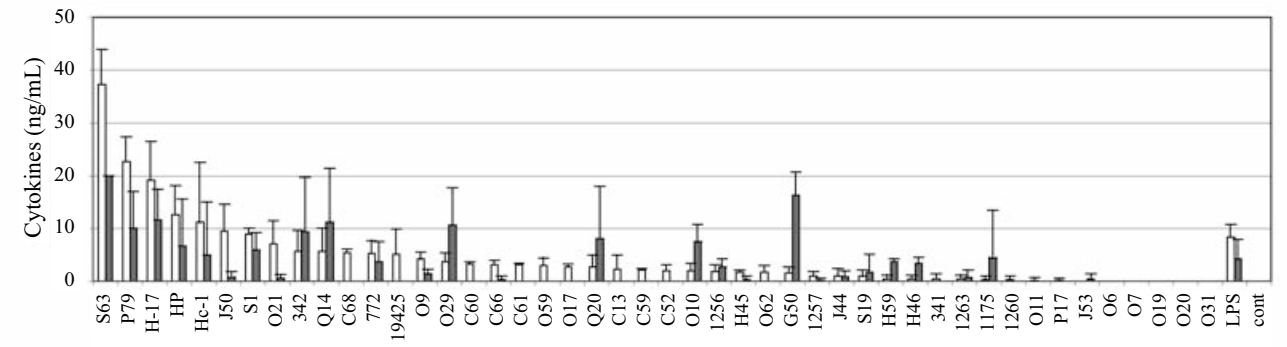

Fig. 1. Effect of *Lactococcus* strains on cytokine production of J774.1 cells

Concentrations of IL-12p40 (white bars) and TNF-α (gray bars) in the culture supernatant after 24 h incubation in the presence of live *Lactococcus* cells (OD_{620}0.25) were measured by ELISA. Strains were represented in the rank order of IL-12p40 induction. Phosphate buffer saline (PBS) was used for negative control (cont) and *E. coli* lipopolysaccharide (LPS) at a concentration of 1 μg/mL was used as a positive control.

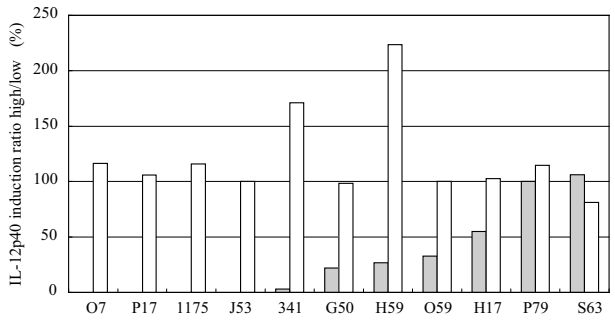

Fig. 2. Effects of dose and heat treatment of the strains on IL-12p40 induction

Concentrations of IL-12p40 in the culture supernatant after 24 h incubation in the presence of *Lactococcus* cells (low dose; OD_{620}0.05, high dose; OD_{620}0.25) were measured by ELISA. The ratio of IL-12p40 concentration in the presence of high dose to that in the presence of low dose was calculated. Gray and white bars represent live and heat-killed cells, respectively.

not induce TNF-α production, suggesting that heat-killed lactococci could be useful for immunomodulation. Recently, Aoki et al. showed that lysozyme-coated *Lactococcus* cells produced by heat treatment with lysozyme were better able to induce IL-12 production in J774.1 cells[3]. Developing means of modifying the IL-12-inducing ability of bacteria could have implications for its exploitation in immunobiotics.

Apoptotic and Cytotoxic effects of *Lactococcus* strains on J774.1

In the course of investigating cytokine induction by *Lactococcus* cells, it was shown that a high dose of live cells of certain strains had an inhibitory effect on J774.1 cells. To investigate the cytotoxic effect of those strains, J774.1 cells treated with *Lactococcus* cells were stained with FITC-conjugated Annexin V and propidium iodide (PI)[23]. Annexin V is known to have a high affinity for phosphatidylserine (PS), which is exposed to the external cellular environment in apoptotic cells[2,27]. Annexin V does not bind to healthy cells, in which PS is located on the cytoplasmic surface of the cell membrane. PI freely permeates dead cells and binds tightly to nucleic acids, resulting in red fluorescence, but does not permeate live or apoptotic cells.

According to Fig. 2, strains O07 and S63 were used as representative strains with high and low cytotoxic effects, respectively. Strain C59 was used as a strain with a low cytotoxic effect and a low ability to induce cytokines (Fig. 1). Microscopic observation indicated that the cell surface of J774.1 cells incubated with strain C59 resembled that incubated with PBS (Fig. 3). Cells were not significantly stained by Annexin V-FITC or PI. The cell membranes of J774.1 cells incubated with strain O07 were shown to be disrupted and the insides were intensively stained by PI, showing damage typical of necrotic cells (Fig. 3). Conversely, those incubated with strain S63 were stained by Annexin V-FITC but not by PI, indicating the localization of PS on the cell surface (Fig. 3). The percentage of

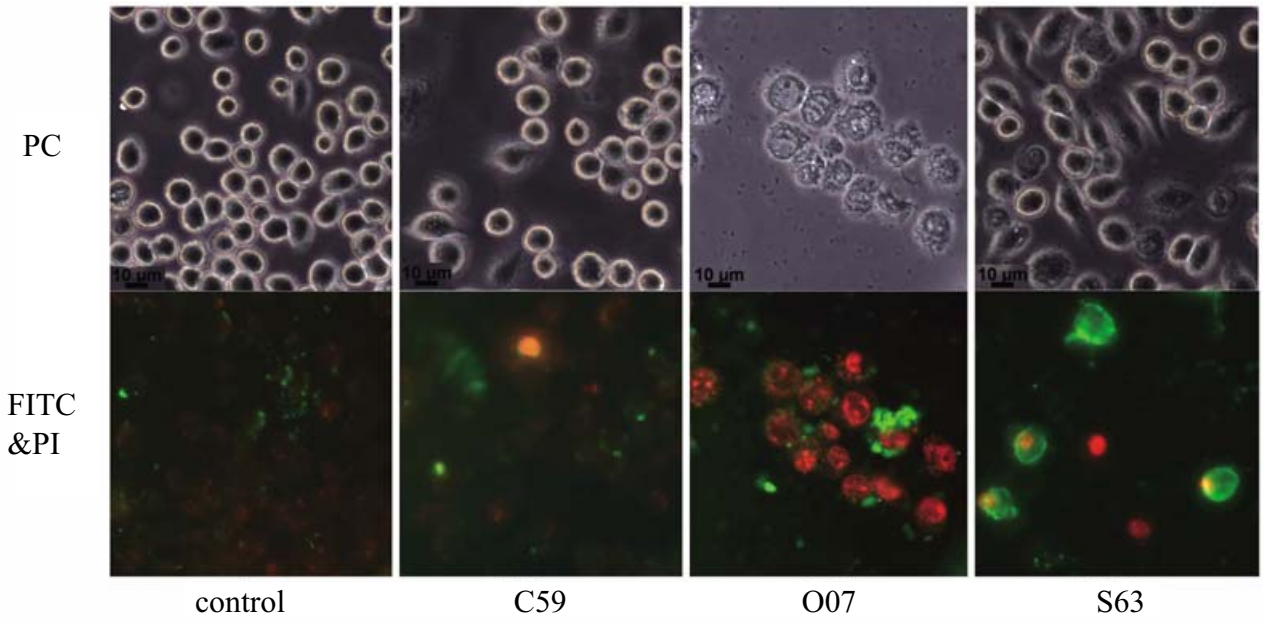

Fig. 3. Phase contrast (PC) and fluorescence microscopy (FITC & PI) of Annexin V-FITC and PI labeled cells in the presence of *Lactococcus* cells (strains C59, S63 and O07)

PBS was used for the negative control, with green and red colors representing Annexin V-FITC and PI, respectively.

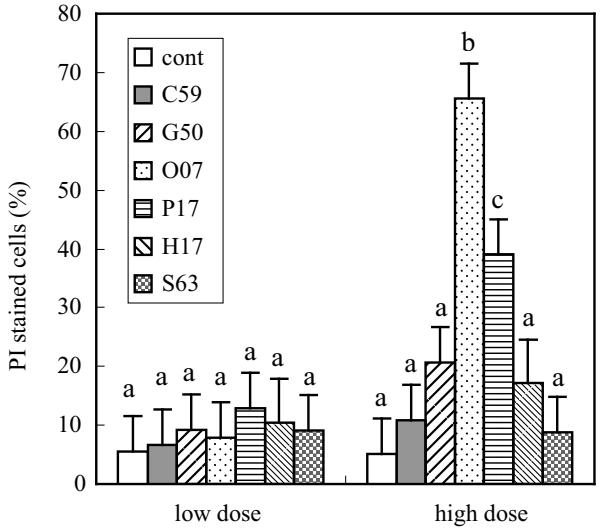

Fig. 4. Percentage of PI-stained cells in J774.1 cells incubated for 24 h in the presence of live *Lactococcus* cells (low; OD_{620}0.05, high; OD_{620}0.25)

PBS was used for negative control (cont). Data are presented as least square means and standard error. Different lowercase letters indicate significant differences ($p<0.01$).

PI-stained cells in the presence of various strains was examined (Fig. 4). When J774.1 cells were incubated with *Lactococcus* strains at a lower concentration (OD_{620} 0.05), the percentage of PI-stained cells did not differ significantly. However, the percentage of PI-stained cells incubated with strains O07 or P17 at a high concentration (OD_{620} 0.25) increased significantly, suggesting that J774.1 cells were killed by these strains.

Phagocytosis of lactococci may act as a stimulus for macrophages to display an apoptotic phenotype in a strain-specific manner. The exposure of PS is one major "eat me" signal for phagocytes of apoptotic and necrotic cells. The strong apoptosis response induced by the pathogens provides a means of defending against gastrointestinal pathogens through the rapid destruction of infected cells aimed at reducing pathogen replication. Since apoptotic cells are engulfed by phagocytes, apoptosis does not usually lead to inflammation, which may be advantageous for the pathogen, in potentially avoiding the triggering and recruitment of host defences[5].

Altonsy et al.[1] showed that probiotic strains, such as *Lactobacillus rhamnosus* strain GG and *Bifidobacterium lactis* strain Bb12, exhibited the capacity to induce apoptosis in the colon cancer cell line and modulate the apoptotic machinery through the mitochondrial pathway as opposed to the FAS receptor route. Apoptotic changes were more pronounced in Caco-2 cells infected with pathogenic enteropathogenic *Escherichia coli* (EPEC) or Vero cytotoxin-producing *E. coli* (VTEC). Infection with strains GG or Bb12 did not cause nuclear disintegration into apoptotic bodies nor did it induce DNA fragmentation, as were clearly seen with EPEC or VTEC infection. In the case of strains of *L. lactis*, DNA fragmentation using the TUNEL assay was insignificant in J774.1 cells incubated with *Lactococcus* cells, even at a high concentration (OD_{620} 0.25) (data not shown).

In Gram-positive bacteria, the capsular polysaccharides (CPS) are omnipresent components of bacteria surfaces, and are considered virulence factors, acting by preventing phagocytosis. Chapot-Chartier et al.[6] showed how the lactococcal surface polysaccharides pellicle, unlike CPS, confers resistance to ingestion by murine macrophages. The susceptibility to phagocytosis of polysaccharide-negative mutant strains indicates that the polysaccharides pellicle can help lactococci escape innate immune defenses. Whether this polysaccharide pellicle is strain MG1363-specific or is general in all *Lactococcus* strains is unclear. The presence or absence of the polysaccharide pellicle or CPS may be one of the determinants of strain-specificity.

Effects of dairy LAB strains on the Th1 cytokine production of splenocytes

We showed how *Lactococcus* cells stimulate the macrophage-like cell line J774.1 in a strain-specific manner. Subsequently, we planned to examine the effect of *Lactococcus* cells in vivo and evaluate whether the oral administration of *Lactococcus* cells would suppress the production of IgE antibody in serum. To select strains for oral administration, we focused on dairy LAB strains because these were eaten for an extended period. The effect of LAB that increases Th1 immune responses has also received considerable attention recently. IL-12 and IFN-γ are key enzymes in Th1 immune responses and capable of suppressing the Th2 response promoting IgE production. To explore dairy LAB strains that increase the Th1 immune responses, the ability of 10 strains in our LAB library and old collection to induce the IL-12p70 and IFN-γ production of splenocytes was examined[28]. Similar to the ability to induce IL-12p40, strain H-17 strongly induced IL-12p70 and IFN-γ among strains of *L. lactis*. Conversely, strain C59 did not induce IL-12p70. Among *Lactobacillus* strains, *Lactobacillus casei* L-14 strongly induced both IL-12p70 and IFN-γ and *Lactobacillus helveticus* Bc-10 induced IFN-γ, but weakly induced IL-12p70.

Oral administration of live *Lactococcus* strain C59 reduced the total IgE antibody production in OVA-sensitized mice[28]

Among 10 strains, two *Lactococcus* strains H-17 and

C59, and two *Lactobacillus* strains L-14 and Bc-10, which showed distinct patterns of IL-12p70 and IFN-γ induction, were selected and their regulatory effect of IgE antibody production was evaluated in vivo[28]. Four strains were orally administered to mice for 7 days, and then the mice were sensitized by twice-repeated OVA and alum precipitate. Total IgE antibody in the serum 7 days after the second OVA injection was examined. As shown in Fig. 5, the administration of C59, which had the lowest ability to induce Th1 cytokines in vitro, significantly reduced the total IgE antibody level compared with that of the control group, while the oral administration of live strain C59 did not affect the OVA-specific IgE antibody levels, OVA-specific serum IgG1 or IgG2a antibody production. Conversely, the oral administration of heat-killed strain C59 did not affect total IgE antibody production, suggesting that heat sensitive ligands may be involved in the mechanism of suppression of IgE antibody production.

Oral administration of strain C59 decreased IL-4 production by splenocytes[28]

To clarify the mechanisms involved in the suppression of IgE antibody production in mice, splenocytes of mice that were orally given PBS or live strain C59 were cultured and cytokine production was examined (Fig. 6). IL-4 production by splenocytes in the presence of anti-CD3 antibodies declined significantly with the oral administration of strain C59. IL-4 promotes IgE class switching and augments IgE secretion from plasma cells[10]. Therefore, the inhibition of IL-4 production may result in the suppression of total IgE antibody production, but not antigen-specific IgE antibody production. This is consistent with the microarray analysis, whereby genes involved in the migration of immune cells were rather down regulated in the ileum of C59-administered mice compared with control mice (manuscript in preparation).

Kimoto et al. reported that the oral administration of the *L. lactis* strain G50 suppressed total and OVM-specific IgE serum levels in OVM-sensitized mice and increased IgG2a antibody production[11], unlike that of strain C59. An explanation for the difference between C59 and G50 remains to be elucidated.

Lactococcus cells pass through the gastrointestinal tract alive[28]

To investigate the persistence of strain C59 in the murine gastrointestinal tract, the recovery of strain C59 in feces after a single oral administration was examined. Since strain C59 has a unique exopolysaccharide synthesis (eps) gene cluster, a partial fragment of the cluster was amplified by PCR and used as a C59-specific probe (manuscript in preparation). When mice received strain C59 suspension (2×10^8 cfu/0.2 mL), 5.0×10^6 cfu and 6.6×10^6 cfu (2 and 5 h after oral administration, respectively) of strain C59 in feces

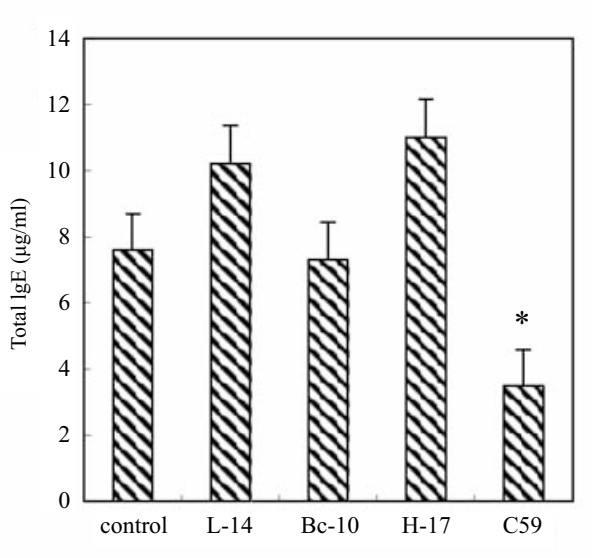

Fig. 5. Effect of oral administration of strains L-14, Bc-10, H-17, and C59 on total IgE antibody production in OVA-sensitized mice

Mice in each group received a bacterial suspension (70 μg dry weight/0.2 mL/day) for 7 days. OVA (100 μg) and alum precipitate (1mg) were intraperitoneally injected on days 14 and 28 for sensitization. On day 35, blood samples were obtained from the tails. The total IgE antibody levels in sera were determined using sandwich ELISA. Data are shown as means ± SE. * indicates a value differing significantly from the control (*$P < 0.05$).

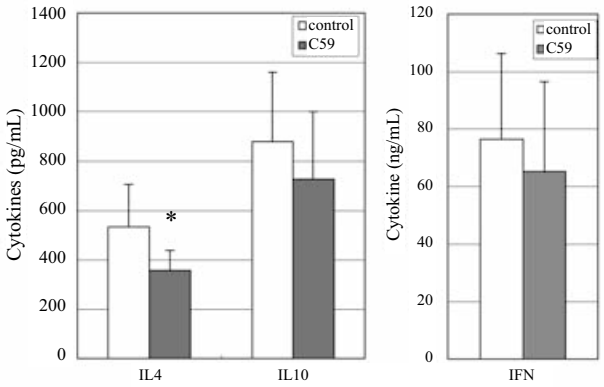

Fig. 6. Effect of feeding of live strain C59 on cytokine production by murine splenocytes stimulated with the anti-CD3 antibody

The production of IL-4, IL-10 and IFN-γ 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 μg/mL) for 24 h. Data are shown as means ± SE. Asterisks indicate values differing significantly from the control (*$P < 0.05$).

during 10 min were detected by colony hybridization (Fig. 7). The number of colonies of strain C59 in feces declined to less than 3×10^5 cfu 8 h after oral administration, and no living cells of strain C59 were detected in feces 24 and 48 h after oral administration (data not shown). No colonies from feces administered with saline (0.85%) were hybridized to the probe. Similarly, when mice received live *L. lactis* strain G50 (2×10^8 cfu), live bacterial cells in feces during 10 min were 2.9×10^6 cfu (0h), 2.6×10^6 cfu (3h), 1.1×10^8 cfu (6h) and 2.6×10^5 cfu (23h) after oral administration. However, no such change in viable cell count was observed in mice given 0.2 mL of PBS. These data suggest that strains of *L. lactis* pass through the gastrointestinal tract 2-6 h after oral administration. In the case of *Lactobacillus*, most of the administered *L. rhamnosus* GG cells were recovered in feces between 6 and 48 h after gavage[16]. Intestinal passage time in strains C59 and G50 was thus faster than probiotic *L. rhamnosus* GG. This means *Lactococcus* strains are delivered live to feces through the intestines, but cannot survive in the intestines, suggesting that it may be difficult for *Lactococcus* strains to affect resident flora.

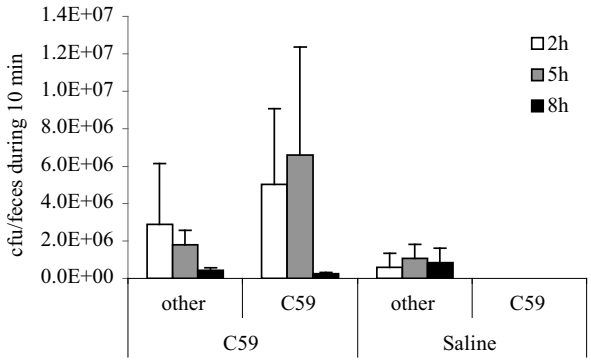

Fig. 7. Number of living strain C59 (gray bar) and others (white bar) in feces after administration of living strain C59

Mice received 0.2 mL of saline (n = 3) or strain C59 (2×10^8 cells) (n = 3). Feces during 10 min were collected 2, 5 and 8 h after oral administration, suspended in PBS and spread on MRS plates. After 2 days at 30°C, the bacterial colonies was counted and transferred to a nylon membrane. The number of strain C59 colonies was also counted after colony hybridization (C59). C59-specific probe (800bp epsH sequence; DDBJ AB99929) was used after labeling with DIG. The number of bacterial colonies other than C59 was represented as (other).

Conclusion

L. lactis is a bacterium "generally regarded as safe", which human beings have eaten since prehistoric times. *L. lactis* is not, however, a human commensal and reported cases of human infection are rare. Conversely, the immunomodulatory effect of *L. lactis* may exceed those of commensal bacteria. Our results suggested that some strains of *L. lactis*, C59 and G50 for example, have the potential to improve the cytokine balance to prevent IgE-dependent allergic diseases. Live, heat-killed and modified *Lactococcus* strains could interact with our immune cells in a strain-specific manner and directly or indirectly affect the immune system. Developing the utilization and assessing the safety of *L. lactis* strains may help improve various immune diseases that have become a social problem.

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