Immunomodulatory Function and Probiotic Properties of Lactic Acid Bacteria Isolated from Mongolian Fermented Milk

Makoto KIMURA1, Kuninaga DANNO2 and Hisako YASUI*

1Sciences of Functional foods (Integrated Department), Graduate School of Agriculture, Shinshu University, Minaminomowa, Kamiina, Nagano 399-4598, Japan
2Horonobe Factory, Snow Brand Milk Products Co., Hokkaido 098-3221, Japan

Received July 14, 2006; Accepted September 15, 2006

We screened lactic acid bacteria which had cellular or humoral immunomodulatory function from 51 strains isolated from Mongolian fermented milk by measuring the inductive activity of IL-12 and INF-γ or IgM, IgG and IgA, respectively. Two strains induced large quantities of IL-12 and INF-γ and had cellular immunomodulatory function, and 3 strains induced large quantities of IgA and had humoral immunomodulatory function. The probiotic properties of these 5 strains were studied by measuring the tolerance to stomach acid, bile and pancreatic fluid and the adherence to human enterocyte-like Caco-2 cells in in vitro tests. Results showed that the N-17 strain (Lactobacillus plantarum), which induced large quantities of IgA, strongly tolerated stomach acid, bile and pancreatic fluid and expressed strong adherence to human enterocyte-like Caco-2 cells. In addition, survival of the N-17 strain during passage through the gastrointestinal tract of mice was examined after 6, 12, 24, 36, 48, 60 and 72 hr of oral administration to mice. Six hr after oral administration, the highest number of viable cells of the strain appeared from feces, and almost all of the administered bacteria were recovered within 48hr. These results suggested that the N-17 strain isolated from Mongolian fermented milk induced large quantities of IgA and showed high survival during passage through the mouse digestive tract. Therefore, this is an excellent probiotic strain which has humoral immunomodulatory function and may be useful as a carrier for the oral vaccine.

Key words: immunomodulatory function; probiotic properties; lactic acid bacteria; Mongolian fermented milk

INTRODUCTION

A lot of studies on the beneficial effects on human health of lactic acid bacteria (LAB) have been reported. These strains of LAB are effective for intestinal disorder and decreasing serum cholesterol, and have anti-mutagenicity activity and immunomodulatory function (12, 13, 17, 28, 32). In recent years, many researchers have been actively studying the immunomodulatory function of LAB, and have reported that LAB prevent or reduce allergies and prevent the host from suffering from various infectious diseases and cancers (4, 8, 10, 13, 21, 26–31, 33). But most of these studies concern the function of starter LAB in commercial fermented milk. In various areas of the world, there are many kinds of traditional fermented milk, and many species and strains of LAB live in the fermented milk. The isolation and classification of this LAB has already been reported (34). However, the physiological properties of this LAB are scarcely researched, and in particular, the investigation of immunomodulatory function is almost never done. It has been thought that LAB isolated from the traditional fermented milk is hardy bacteria, because they grow in a severe environment. Thus, it is expected that the physiological function, probiotic activity, immunological activity, etc., of this LAB is stronger than that of starter LAB of commercial fermented milk.

Therefore, we examined the immunomodulatory function of LAB isolated from traditional Mongolian fermented milk using the mouse spleen cell culture method. The cellular immunomodulatory function was determined by measuring the inductive activity of interleukin(IL)-12 and interferon(IFN)-γ, and the humoral immunomodulatory function was determined by measuring the inductive activity of immunoglobulin(Ig) M, IgG and IgA. IL-12 is secreted by an activated macrophage (MΦ) and activates type I helper T (Th1) cells and natural killer (NK) cells. IFN-γ is secreted by activated Th1 cells and NK cells. MΦ, NK cells, T cells and cytokines produced by these cells regulate cellular immunity (18, 20) and play a part in anti-cancer function, prevention from infection and anti-allergy function (21, 27, 29, 33). IgM and IgG are important antibodies in serum and IgA is an important antibody in mucosal immunity. IgA prevents the host from pathogenic microbial infection in mucosal sites (22, 31). We
selected 2 strains with cellular immunomodulatory function and 3 strains with humoral immunomodulatory function from 51 strains isolated from traditional Mongolian fermented milk.

In recent years, probiotic activity of LAB has been emphasized (19). Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance (3). Probiotic activity of starter LAB from commercial fermented milk has already reported (1, 7, 9, 15, 24). But studies on the probiotic activity of LAB from traditional fermented milk are very rare. The probiotic strains should be required properties to survive during passage through the gastrointestinal tract and to stay a long time in the intestines (19, 25). Therefore, they must have the tolerance to digestive fluid such as the stomach acid, bile and pancreatic fluid and they need to have high adhesive properties to the intestinal epithelial cells or the intestinal mucus (1, 7, 15). We tested the probiotic activity of 5 strains with immunomodulatory function. In in vitro tolerance tests to digestive fluids, the viable cells after culture with and without digestive fluids were counted and compared between both cultures (9, 24). In addition, the adhesive property of LAB was measured using enterocyte-like Caco-2 cells (Caco-2 cells). In the next experiment, survival during passage through the gastrointestinal tract of a strain having high probiotic activity in in vitro tests was tested using fecal samples after oral administration to mice (11).

In this study, we found some strains with immunomodulatory function, and one strain (N-17 strain) had high IgA inductive activity, strong tolerance to stomach acid, bile and pancreatic fluid and strong adhesive activity to Caco-2 cells in in vitro tests. This strain had the ability to pass live through the gastrointestinal tract after oral administration to mice.

MATERIALS AND METHODS

Bacterial strains

Fifty-one strains isolated from Mongolian fermented milk were used. The strains were the following species: 22 strains of Lactococcus (Lc) lactis subsp. lactis, 4 strains of Lactobacillus plantarum, 10 strains of Lc. lactis subsp. cremoris, 1 strain of Leuconostoc (Leu) mesenteroides subsp. mesenteroides, 13 strains of Leu. mesenteroides subsp. dextranicum and 1 strain of Weissella viridescens. These organisms were incubated in de Man, Rogosa and Sharpe (MRS) broth at 37°C for 48 hr. For the immunological experiment, the cultured organisms were harvested by centrifugation at 2,000 rpm for 20 min and washed three times with phosphate buffer saline (PBS) and freeze-dried. Organisms treated at 121°C for 10 min were used. For the experiment concerning probiotics properties, the organisms cultured in MRS broth and washed with PBS were used.

Animals

For the immunomodulatory function assay, female BALB/c mice were purchased from CLEA (Tokyo, Japan) and were used at 8 weeks of age. For the bacterial survival assay after oral administration, female BALB/c mice were purchased from SLC (Shizuoka, Japan) and were used at 11 weeks of age. The animal experiments in the present study were followed the guideline for regulation of animal experimentation of the Faculty of Agriculture, Shinshu University.

Cell cultures for cytokine and antibody production

The spleen cells were isolated as single-cell-suspension from the spleens of mice. After depletion of erythrocytes, the cells (5 × 10^5 cell/well) were cultured with 10 μg/ml (for cytokine induction) or 100 μg/ml (for antibody induction) heat-killed bacterial strains in a 200 μl RPMI-1640 medium (Sigma, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin in a 96 well culture plate (Nunc, Denmark), at 37°C in 5% CO₂ incubator. Supernatants were collected at days 3 and 7 for determination of cytokine and antibody levels, respectively.

Measurement for cytokines and antibodies

Determination of IFN-γ and IL-12 was performed using the sandwich ELISA method of Shida et al. (26) with modification. Goat anti-mouse IFN-γ capture antibody (Biosource, USA) or goat anti-mouse IL-12 capture monoclonal antibody (Pharmingen, USA) were coated on the wells of a 96-well ELISA plate (Nunc, Denmark) using a carbonate buffer with pH9.6. After blocking the unoccupied sites on the plate with bovine serum albumin (BSA), the test samples and standard recombinant mouse (r)INF-γ (Biosource, USA) or rIL-12 (Pharmingen, USA) were added. As the detection of cytokines, biotinylated anti-mouse INF-γ (Biosource, USA) or anti-mouse IL-12 (Pharmingen, USA) were used, respectively. Streptavidin-horseradish peroxidase conjugate was added to each well. 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate (Sigma, USA) was added to the wells and then the reaction was stopped by 1M H₂SO₄. The absorbance of the contents of the wells was read at 450 nm on a Microplate Reader (Bio-Rad Laboratories, USA).
**Culture of the Caco-2 cells**

The Caco-2 cell line isolated from a human colon adenocarcinoma was obtained from the Riken Cell Bank (Japan). Cells were routinely grown at 37°C in 5% CO2 incubator in Dulbecco modified Eagle’s minimal essential medium (D-MEM; Gibco, USA) containing 10% FCS, 1% antibiotic antymycotic solution (100×) (Sigma, USA) and 1% non essential amino acids (Gibco, USA). For the adhesion assay, monolayers of Caco-2 cells were prepared on 8 well-chamber slides (Falcon, USA) and were used at late postconfluence after 15 days of culture. The medium was replaced every other day.

**In vitro adhesion assay**

Adhesion assay was performed by the method of Lee et al. (15) with modification. Monolayers of Caco-2 cells were washed once with PBS. Bacterial suspension (about 5 × 10^7 bacteria / 0.5 ml) was added to each well, which was incubated at 37°C in 5% CO2 incubator for 1 hr. Non-adhered bacteria in each well were washed three times with PBS and Caco-2 cells and adhered bacteria in each well were harvested with trypsin solution and resuspended with PBS. Then the number of adhered bacteria was measured with the colony count method on MRS agar plate. Adhesive activity was expressed as the number of bacteria adhered to 1 cm^2 of Caco-2 cells.

**Resistance to stomach acid, bile and pancreatic fluids**

The method of Ronka et al. (24) was used with modification. In the test for tolerance to stomach acid, MRS broth adjusted to pH3.0 with 3N HCL containing 0.3% pepsin (Wako, Japan) was used. MRS broth with pH6.2 without supplements was used as the control medium. Each bacterial strain was inoculated at 10% inoculum size in the test or control medium. In the test for tolerance to bile, MRS broth supplemented with 0.3% oxygall (Wako, Japan) was used. MRS broth without supplements was used as control medium. Each bacterial strain was inoculated at 1% inoculum size in the test or control medium. In the test for tolerance to pancreatic fluid, MRS broth adjusted with 180 mM NaHCO_3 to pH8.0 containing 1.9mg/ml pancreatin (Sigma, USA) was used. MRS broth with pH6.2 without supplements was used as control medium. Each bacterial strain was inoculated at 10% inoculum size in the test or control medium. These cultures were incubated at 37°C for 24 hr. Survival of bacteria in each culture was examined by the colony count method on MRS agar plate. The number of colonies was measured after incubation at 37°C for 48 hr.

**Determination of IgM, IgG and IgA** was performed by the sandwich ELISA method of Yasui et al. (32) with modification. Goat anti-mouse IgM, IgG, or IgA (Cappel, USA) were coated on the wells of a 96-well ELISA plate (Nunc, Denmark) using a carbonate buffer with pH9.6. After blocking the unoccupied sites on the plate with BSA, the test samples and standard rIgM, rIgG, or rIgA (Sigma, USA) were added. For the detection of antibodies, peroxidase-labeled anti-IgM (Zymed, USA), IgG or IgA (Cappel, USA) were used, respectively. Then o-phenylenediamine solution (0.4 mg of citrate buffer per ml, pH5.0) containing 0.01% H_2O_2 was added to each well. The reaction was stopped by 1M H_2SO_4. The absorbance of the contents of wells was measured at 495 nm with a Microplate Reader (Bio-Rad Laboratories, USA).

**Detection of N-17 strain from feces**

This study was performed according to the method of Kimoto et al. (11) with modification. Rifampicin-resistant variants of the N-17 strain (RRVN-17) were obtained from MRS agar plates containing 0.1mg/ml of rifampicin. RRVN-17 was cultured in MRS broth containing 0.1 mg/ml of rifampicin at 37°C for 24 hr. Then RRVN-17 was washed once with PBS and resuspended in 10%(w/v) nonfat milk. Preparation of RRVN-17 was administered orally as suspension to mice (n=3) by a single dose of 1 × 10^9 CFU in 200 μl per mouse. Feces were collected into microtubes, and weighed. One milliliter of PBS was added to 0.1 g of feces and homogenized. Quantitative analysis was carried out to determine the presence of RRVN-17 in feces before administration (0h) and from each sampling time as follows: 6, 12, 24, 36, 48, 60, and 72 hr. The number of RRVN-17 was tested on MRS agar plate containing 0.1 mg/ml of rifampicin. The plates were incubated at 37°C for 48 hr, and then the number of colonies was counted. To determine wether the original N-17 strain and the RRVN-17 strain administered orally were same strains as the RRVN-17 strain recovered from feces, they were checked with an API-system (BioMerieux, France).

**Statistical analysis**

Difference in adhesion to Caco-2 cells among various strains was analyzed with the Tukey multiple comparison test. Differences in the number of viable bacteria between the control medium and the test medium containing stomach acid, bile or pancreatic fluid were analyzed by a paired t-test. Values of p<0.05 were considered significant.
RESULTS

Selection of LAB that have high immunomodulatory function

Th1 cells produce various cytokines and activate cellular immunity, and as a result, allergies, cancer and infectious diseases are suppressed. Th1 cells are activated by IL-12, which is produced by MΦ. We examined the cellular immunomodulatory function of 51 strains of LAB isolated from Mongolian fermented milk using the mouse spleen cell culture method, and found some strains that induce large quantities of IL-12 and IFN-γ (Fig. 1A, B). T-120 (Lc. lactis subsp. lactis) and T-130 (Lc. lactis subsp. lactis) strains, which induced IL-12 very highly, were thought to be an identical strain, because they were the same species and had similar properties in induction of tested cytokines and antibodies. But the perfect identity between T-120 and T-130 strains must be judged by pulsed-field gel electrophoresis (PFGE) pattern assay. T-120 (Lc. lactis subsp. lactis), T-50 (Lc. lactis subsp. cremoris) and T-20 (Lc. lactis subsp. lactis) strains highly induced both IL-12 and INF-γ. Thus, it was found that these strains greatly modified cellular immunity.

IgM and IgG are the important antibodies for prevention from pathogenic microorganisms in serum. IgA is an important antibody for prevention from foreign antigen on mucosal sites. Therefore, we measured the induction of IgM, IgG and IgA by LAB isolated from Mongolian fermented milk (Fig. 2A, B, C). The inductive activity of antibodies of each strain differed according to antibody class (IgM, IgG and IgA). As IgA is an immunoglobulin which provides an immunological barrier against foreign matter by preventing the absorption of such material into the mucosal epithelium and the penetration of such material into the body, we aimed at the strain which induced a large quantity of IgA. The N-1 (Leu. mesenteroides subsp. mesenteroides), N-17 (Lc. lactis subsp. lactis) and T-20 (Lc. lactis subsp. lactis) strains induced large quantities of IgA.

Finally, five strains that had immunomodulatory function were selected (Table 1). T-120 (Lc. lactis subsp. lactis) and T-50 (Lc. lactis subsp. cremoris) strains induced large quantities of IL-12 and showed high cellular immunomodulatory function. N-1 (Leu. mesenteroides subsp. mesenteroides), N-17 (Lc. lactis subsp. lactis) and T-20 (Lc. lactis subsp. lactis) strains induced large quantities of IgA and showed high humoral immunomodulatory function. In the next experiment, we

Fig. 1. Difference in IL-12 (A) and IFN-γ (B) induction among 51 strains isolated from Mongolian fermented milk. Fifty-one strains were added at 10 μg / ml each to cultured spleen cells. Three days later, IL-12 and IFN-γ levels in the culture supernatants were measured by ELISA.
tested the probiotic properties of these five strains.

*Probiotic properties of 5 strains that have high immunomodulatory function*

Tolerance to stomach acid, bile and pancreatic fluid in *in vitro* tests is expected to predict the survival of these strains in the conditions of the gastrointestinal tract. The N-17 strain maintained their viability in MRS broth with pH3.0 for duration of the 3 hr test period. But the viability of the four other strains in MRS broth with pH3.0 was significantly lower than that with pH6.2 (control medium) (Fig. 3A). Incubation in the growth medium containing bile had no effect on the N-17 strain, but had an effect on the four other strains (Fig. 3B). The N-17 strain showed tolerance to bile. Incubation in the growth medium containing pancreatic fluid did not effect
the four strains, and increased the number of bacteria leaving the strain (N-17 strain), significantly (Fig. 3C). Thus, the N-17 strain showed a strong tolerance to stomach acid, bile and pancreatic fluids.

Five strains were examined for their ability to adhere to Caco-2 cells (Fig. 4). Both the N-1 and N-17 strains expressed significantly stronger adhesion than the T-20, T-50 and T-120 strains. Finally, the N-17 strain was selected as the microorganism having the strongest probiotic property.

Survival of the N-17 strain during passage through the mouse digestive tract

The N-17 strain showed strong probiotic properties in the results of in vitro tolerant tests in the conditions of the upper part in the gastrointestinal tract and in vitro adhesive assay to Caco-2 cells. Consequently, this strain was used in the in vivo survival test to pass through the

Fig. 3. Sensitivity to stomach acid, bile and pancreatic fluids of the strains screened as LAB with immunomodulatory function. Five strains were grown in a test medium (stomach acid (A), bile (B) or pancreatic fluid (C)) (■) and control medium (□). Three hours later, the viable bacteria from the culture medium was measured by the colony count method on MRS agar plate. Results are expressed as the mean ± SD of the three independent experiments. Significant difference from the control: **p<0.01, *p<0.05.

Fig. 4. Adhesive activity of the 5 strains to Caco-2 cells. Each viable 5 strain bacteria was added to monolayers of Caco-2 cells, which were then cultured at 37°C in 5% CO₂ incubator for 1 hr. The number of adhesive bacteria was measured by the colony count method on MRS agar plate and was shown as the number of bacteria adhered to 1cm² of monolayers of Caco-2 cells. Results are expressed as the mean ± SD of the three independent experiments. Means with different letters are significantly different. a, b <0.05.
mouse gastrointestinal tract. Viable cells through the mouse gastrointestinal tract were investigated by analyzing the recovery of the microorganisms in fecal samples (Fig. 5). Six hours after oral administration, the RRVN-17 strain appeared at a maximum dose level of $10^9$ CFU / g in feces, then 48 hr or 72 hr after oral administration, RRVN-17 appeared at levels of $10^3$ or $10^2$ CFU / g in feces, respectively. On the API-system assay, the original N-17, the RRVN-17 administered orally and the RRVN-17 strains recovered from the feces had the same properties and were the same strain. Finally, the microorganisms administered orally were almost all recovered in the feces.

**DISCUSSION**

Recently, many researchers have reported that some strains of starter LAB of commercial fermented milk have an immunomodulatory function and can prevent or improve allergic diseases, infectious diseases and cancer (2, 4, 8, 12–14, 21, 22, 26, 27, 29–33). The probiotic activity of some strains of the starter LAB have also been demonstrated in *in vitro* tolerance tests to digestive fluids, adhesive tests to Caco-2 cells or *in vivo* survival tests through the gastrointestinal tract (1, 7, 9, 11, 15, 24).

In this study, we found some strains with strong immunomodulatory function from 51 strains of LAB in Mongolian fermented milk, which was the typical traditional fermented milk. The immune response to LAB was not different between live and heat-killed bacteria (data not shown). Thus, we used heat-killed bacteria for assay of immune activity, because they can be preserved for long time.

We measured IL-12 and IFN-γ as the markers of cellular immunomodulatory function and screened 2 strains with cellular immunomodulatory function, particularly the inductive activity of IL-12 (Fig. 1, Table 1). IgE is involved in mediating many allergic diseases. The production of IgE is promoted by Th2 cells and their cytokines, such as IL-4 and IL-5, while IFN-γ secreted by Th1 cells suppresses the response of Th2 cells and can inhibit IgE production (18). The balance of responding Th1 and Th2 cells is critical for regulating IgE production. Several studies have shown that a polarized Th2 response is often observed in patients with allergies (23). Thus, enhancing a Th1-type immune response is expected to be beneficial for the treatment of allergic diseases such as anaphylaxis shock, hay fever, and atopic dermatitis (4, 8, 10, 20, 21, 26, 27). Thus, 2 strains inducing large quantity of IL-12 and IFN-γ are expected to have anti-allergy activity. In addition, their strains may have preventive effects on infection and cancer.

IgA is an important antibody in intestinal mucosal immunity (5, 22), and IgM and IgG are the main antibodies in the serum, which prevent the host from pathogenic microorganisms. We have already reported that *Bifidobacterium breve* YIT4064 activated the humoral immune system and augmented anti-rotavirus IgA production or anti-influenza virus IgG production and protected against rotavirus infection or influenza, respectively (30, 31, 33). Therefore, IgM, IgG and IgA were measured as the markers of humoral immunomodulatory function. We screened 3 strains with humoral immunomodulatory function, particularly the inductive activity of IgA (Fig. 2, Table 1). These strains are expected to activate the humoral immune system on mucosal sites and prevent the host from pathogenic microbial infection, as well as suppress the absorption of allergens into the intestine (2, 6, 22).

In the next study, we evaluated the probiotic properties of 5 strains with immunomodulatory function. In *in vitro* assay, we tested the tolerance to gastric acid, bile, and pancreatic fluids and the adhesion to Caco-2 cells of 5 strains with immunomodulatory function. The N-17 strain had strong tolerance to all digestive fluids and strong adhesion to Caco-2 cells (Figs. 3 and 4). In addition, we tested the survival of the N-17 strain through the gastrointestinal tract with an *in vivo* test. To distinguish administered N-17 strain from resident LAB, rifampicin-resistance was added to N-17 strain (RRVN-17 strain). After oral administration of the RRVN-17 strain, the viable cells from the RRVN-17 strain in feces of mice were measured using MRS agar plate containing rifampicin (Fig. 5). Six hours later, viable cells from the RRVN-17 strain were detected at a level of $10^9$ CFU / g.
in feces. The results indicated that the strain reached the intestine alive. It has been suggested that probiotic bacteria cannot influence the environment of the intestinal tract unless their population reaches a certain minimum level between $10^6$ and $10^8$ CFU/g of intestinal content (16). It seems feasible to obtain a high viable level of the N-17 strain in the human intestine. If the N-17 strain is administered daily, a considerable population of viable cells may exist continuously in the intestine. The safety of probiotic strains has been of prime importance (25) and the N-17 strain is thought to be safe, since this strain has been taken as traditional fermented milk in Mongolia for long time. The N-17 strain may be a promising candidate for a probiotic strain with humoral immunomodulatory function and be useful as carrier of oral vaccine, since this strain survives well in the gastrointestinal tract and has adjuvant activity inducing large quantity of IgA. The development of an oral vaccine using the N-17 strain as the vaccine delivery vehicle is currently being investigated.

**REFERENCE**


87–95.