Effects of *Lactobacillus kefiranofaciens* M1 isolated from kefir grains on enterohemorrhagic *Escherichia coli* infection using mouse and intestinal cell models

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ABSTRACT

A potential probiotic strain, *Lactobacillus kefiranofaciens* M1, was previously isolated from kefir grains, which are used to manufacture the traditional fermented drink kefir. The aim of this study was to investigate the effects of *Lb. kefiranofaciens* M1 on enterohemorrhagic *Escherichia coli* (EHEC) infection, using mice and intestinal cell models. BALB/c mice were daily administrated with either phosphate buffered saline or *Lb. kefiranofaciens* M1 at 2×10^8 cfu/mouse per day intragastrically for 7 d. Intragastric challenges with EHEC (2×10^9 cfu/mouse) were conducted on d 0, 4, and 7 after treatment. Administration of *Lb. kefiranofaciens* M1 was able to prevent EHEC infection-induced symptoms, intestinal damage, renal damage, bacterial translocation, and Shiga toxin penetration. Furthermore, the mucosal EHEC-specific IgA responses were increased after *Lb. kefiranofaciens* M1 administration in the EHEC-infected mouse system. Additionally, in vitro, *Lb. kefiranofaciens* M1 was shown to have a protective effect on Caco-2 intestinal epithelial cells and Caco-2 intestinal epithelial cell monolayers; the bacteria limited EHEC-induced cell death and reduced the loss of epithelial integrity. These findings support the potential of *Lb. kefiranofaciens* M1 treatment as an approach to preventing EHEC infection and its effects.

Key words: probiotic, *Lactobacillus kefiranofaciens* M1, kefir, enterohemorrhagic *Escherichia coli*

INTRODUCTION

Outbreaks of enterohemorrhagic *Escherichia coli* (EHEC) infection are a severe epidemiological problem worldwide. By 2011, authorities have reported 22 fatalities and ca. 2,000 infections related to EHEC, with steadily increasing numbers. The transmission

routes of EHEC are associated with the ingestion of contaminated food, including beef, vegetables, fruit, and water (Serna and Boedeker, 2008; Mohawk and O'Brien, 2011). Particularly, EHEC serotype O157:H7 has been found to be involved in many large-scale food-borne infectious disease outbreaks recently (Lim et al., 2010). Enterohemorrhagic *E. coli* are defined as strains of *E. coli* that cause hemorrhagic colitis and possess the ability to produce Shiga toxin (Stx), which can damage intestinal epithelial cells directly (Kaper et al., 2004). Shiga toxins are also able to attack renal endothelial cells and induce hemolytic uremic syndrome (Hodges and Gill, 2010; Lim et al., 2010), which may cause acute renal failure in humans (Tarr et al., 2005). No effective treatment for EHEC infection exists yet. Only supportive treatments, such as intravenous supplementation with saline or isotonic crystalloid (Tarr et al., 2005), can be provided clinically. Although several novel strategies have been proposed for the treatment of EHEC infection, including the application of antibiotics, the use of Stx-binding agents, treatment with antithrombotic agents, and vaccination, none of them have been successfully applied in the field due to their limitations in terms of clinical practice. Thus, searching for an efficient way to prevent or ameliorate EHEC infection is urgently needed (Tarr et al., 2005; Serna and Boedeker, 2008).

Probiotics are live microbes that are able to improve human health, including the amelioration of gastrointestinal disorders. Certain species or strains of probiotics have been shown to have antiinfective or antibacterial ability against various intestinal pathogens in vitro and in murine models via various putative mechanisms (Gareau et al., 2010). Among these strains, several lactobacilli and bifidobacteria have been shown to have potential in preventing EHEC infection and when treating disease caused by EHEC (DuPont and DuPont, 2011). It has been suggested that the possible mechanism by which these bacteria act against EHEC may involve alterations in the intestinal microbiota after consumption of the probiotic strain (DuPont and DuPont, 2011). However, no direct evidence has shown

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why alternation of microbiota by administration of probiotics could reduce EHEC infection.

Kefir, which is made by inoculating kefir grains into milk, is an alcoholic fermented dairy drink that has multiple health-promoting properties (Guzel-Seydim et al., 2011). The microorganisms presenting in kefir grains, mainly consisting of lactic acid bacteria and yeasts, might play a key role in its functionalities. The composition of kefir grains is influenced by many environmental factors (Chen et al., 2008; Wang et al., 2008; Guzel-Seydim et al., 2011). Santos et al. (2003) indicated that some *Lactobacillus* spp. isolated from European kefir grains show in vitro antimicrobial activity against *E. coli*. However, the inhibition and prevention of EHEC or other pathogenic *E. coli* infection in vivo by kefir itself or by any microbe isolated from kefir grains has not been reported.

In our previous study, several potential probiotic strains were isolated from Taiwanese kefir grain (Chen et al., 2008). Among these strains, *Lactobacillus kefiranofaciens* M1 came to our attention. This strain has been shown to demonstrate effective immunomodulating, antiallergic effects, and antiasthmatic activity, both in vitro and in vivo (Hong et al., 2009, 2010, 2011). It is worth noting that the heat-killed *Lb. kefiranofaciens* M1 also demonstrated strong antiallergic effects. Most recently, we have also shown that *Lb. kefiranofaciens* M1 is able to strengthen the intestinal barrier and prevent chemical-induced colitis in a dextran sodium sulfate (DSS)-mouse model and that it does this via a toll-like receptor (TLR)2-dependent pathway (Chen et al., 2012; Zhang et al., 2012). We further analyzed the cell wall composition and found that *Lb. kefiranofaciens* M1 possessed glycosyltransferase (GlcNAc; 64%), glucose (26%), and galactose (10%), which was very different from type strain *Lb. kefiranofaciens* BCRC 16059 (our unpublished data). According to these findings, *Lb. kefiranofaciens* M1 is a unique strain. These features make *Lb. kefiranofaciens* M1 a perfect candidate probiotic bacterium for protecting against enteric pathogen infection. Therefore, the objective of this study was to investigate the efficacy of *Lb. kefiranofaciens* M1 on a preventive measure against EHEC O157:H7 infection using an in vivo mouse and an in vitro cell model. This study might also provide the scientific evidence explaining the certain functionalities involving in kefir milk.

MATERIALS AND METHODS

Lactobacillus kefiranofaciens M1 Sample Preparation

Lactobacillus kefiranofaciens M1 was isolated and identified previously (Chen et al., 2008). *Lactobacillus kefiranofaciens* M1 was cultured in de Man, Rogosa,

and Sharp (MRS) broth (Difco Laboratories Inc., Detroit, MI) at 37°C and was harvested during log phase by washing and resuspending 3 times in PBS (HyClone Laboratories Inc., South Logan, UT). After washing, the bacterial cells were resuspended in PBS and adjusted to the indicated concentrations. Heat-inactivated *Lb. kefiranofaciens* M1 was prepared by heating at 85°C for 40 min as described previously (Hong et al., 2011).

EHEC O157:H7 Preparation

Enterohemorrhagic *E. coli* O157:H7 ATCC 35150 was obtained from the American Type Culture Collection (Manassas, VA). Enterohemorrhagic *E. coli* O157:H7 was cultured in tryptic soy broth (TSB; Acumedia, Lansing, MI) at 37°C for 12 h and was harvested by washing and resuspending 3 times in PBS (HyClone Laboratories Inc.) to the indicated concentrations.

EHEC Infection Model

The EHEC infection scheme in mice was modified from the one published by Mohawk et al. (2010). Eight-week-old specific pathogen-free female BALB/c mice (National Laboratory Animal Center, Taipei, Taiwan) were maintained in a standard cage environment at 23 to 25°C while being exposed to a 12-h light and dark cycle. All experiments were approved by Institutional Animal Care and Use Committee of National Taiwan University (Taipei, Taiwan) and performed in accordance with guidelines for animal care of the National Science Council in Taipei, Taiwan (IACUC approval number NTU-100-EL-100). Mice with similar BW were separated to give 6 to 9 mice per group. These groups were administrated daily with either PBS (HyClone Laboratories Inc.) or 2×10^8 cfu of *Lb. kefiranofaciens* M1/mouse per day intragastrically for 7 d. This was followed by intragastric challenges with EHEC O157:H7 (2×10^9 cfu/mouse), which were conducted at d 0, 4, and 7 after the end of *Lb. kefiranofaciens* M1 treatment. Food intake was recorded during the infection period. The mice were killed at d 9 by cervical dislocation, which was followed by organ collection. The weights of the cecum, spleen, and kidneys were recorded.

Fecal Bleeding Assessment

The feces were collected from the mice and immediately the amount of occult blood in feces was measured using a Hemocult Sensa assay (Beckman Coulter Inc., Brea, CA; Zhang et al., 2012).

Analysis of EHEC O157:H7 Amount in Organs and Blood

Liver, spleen, and blood samples were collected using an aseptic procedure in a laminar flow cabinet. Blood

samples were collected by submandibular bleeding and cultured in brain heart infusion (BHI; Acumedia) overnight and then plated on EHEC O157-selective agar CHROMagar O157 (CHROMagar, Paris, France) for colony enumeration. Alternatively, tissue samples from the various organs were homogenized in 0.1% peptone water using a gentleMACS Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and then plated on EHEC O157 selective agar CHROMagar O157 (CHROMagar) for colony enumeration.

Histological Evaluation

Histological changes affecting the distal ileum, as well as the colon, cecum, and kidney, were observed after hematoxylin and eosin staining. The feces in the intestines and the contents of the cecum were removed by flushing with ice-cold PBS (HyClone Laboratories Inc.). Intestinal fragments that were 1 cm long were isolated and then washed with ice-cold PBS (HyClone Laboratories Inc.). The washed intestinal fragments and intact kidneys were immediately immersed in 10% histological grade phosphate-buffered formalin (Mallinckrodt Chemical Ltd., Derbyshire, UK) for fixation. The fixed tissue and organ samples were dehydrated in ethanol and then embedded in paraffin wax, which was followed by sectioning (5- μ m thickness) and staining with hematoxylin and eosin stain. Histological evaluation was carried out using the intestine scoring system developed by Dieleman et al. (1998) and each sample was evaluated by well-trained histologists. Briefly, the histological score of each intestinal tissue section was based on the level of tissue inflammation, the extent of tissue involved in the inflammation, the amount of tissue regeneration present, and the amount of crypt damage that had occurred.

Measurement of Immunoglobulin Production

The levels of total IgG, IgM, and IgA in the feces and blood were measured using mouse IgG, IgM, and IgA ELISA quantification kits (Bethyl Laboratories, Montgomery, TX), respectively. Enzyme-linked immunosorbent assays were performed using the standard protocol as recommended by the manufacturer. The levels of anti-*E. coli* O157:H7-specific IgG, IgM, and IgA in sera or feces were also measured using an ELISA-based method (Shu and Gill, 2002; Gagnon et al., 2006). Briefly, formalin-killed EHEC O157:H7 (5×10^6 cells) was coated onto a MaxiSorp microplate (Nunc, Thermo Fisher Scientific, Waltham, MA) in 100 μ L of PBS (pH 7.3) at 4°C overnight. After coating, the plates were washed 4 times with 0.1% Tween 20

in PBS (PBST) and then blocked by blocking buffer (0.1% Tween 20 and 3% BSA in PBS) for 1 h at room temperature. Supernatants obtained from the various fecal suspensions (100 mg/mL) in dilution buffer (0.5% BSA in PBST) and sera obtained from the blood samples were added to the coated wells and incubated for 2 h at room temperature. After incubation, the plates were washed 4 times with PBST. This was followed by adding biotinylated anti-mouse IgG, IgM, or IgA antibodies (Bethyl Laboratories) for 2 h at room temperature. The plates were then washed 4 times with PBST, which was followed by treatment with streptavidin-horseradish peroxidase (BioSource, Invitrogen Corp., Carlsbad, CA) for 30 min at room temperature. Next, the plates were washed again and then finally treated with 3,3',5,5'-tetramethylbenzidine (Clinical Science Products Inc., Mansfield, MA) for 30 min at room temperature. This reaction was stopped by adding 1 *N* H₂SO₄. The results were collected by measuring absorbance at 450 nm using an ELISA microplate reader (VMax; Molecular Devices LLC, Sunnyvale, CA).

Measurement of Stx Production

The level of Stx-1 and Stx-2 in the sera and fecal supernatants (100 mg/mL) were measured using a Prolisa EHEC EIA assay kit (Pro-Lab Diagnostics, Toronto, ON, Canada) according to the manufacturer's instructions. The level of Stx in the EHEC cultured medium was measured after 10-fold dilution in deionized distilled H₂O.

Assessment of Intestinal Epithelial Cell Viability

Human colonic epithelial cell line Caco2-C2BBel was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp.), 50 μ g/mL of penicillin, 50 μ g/mL of streptomycin sulfate, and 100 μ g/mL of neomycin sulfate (Invitrogen Corp.) in a humidified atmosphere of 5% CO₂ at 37°C. For the cell viability test, Caco-2 cells were seeded onto a 96-well plate (10^4 cells/well) and cultured overnight. After that, the cells were treated with *Lb. kefiranoferiens* M1 at indicated concentrations (2×10^4 or 10^5 cfu/well) for 24 h, which was followed by challenging with EHEC (10^4 cfu/well) for 24 h. For the lactose dehydrogenase (LDH) assay (BioVision Inc., Milpitas, CA), the cell medium was collected after treatment and filtered using a 0.22- μ m-pore-size membrane (Pall Corp., Port Washington,

NY); the sample was then analyzed to obtain cell viability according to the manufacturer's instructions. For the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, St. Louis, MO) assay, the remaining cells were washed with 100 μ L of PBS (HyClone Laboratories Inc.) 3 times and then incubated with 1 mg/mL of MTT in cell medium for 2 h at 37°C in a 5% CO₂ incubator; at the end of 2 h, the MTT medium was removed and 150 μ L of dimethyl sulfoxide (Sigma) was added to each well to dissolve and resuspend the formazan crystals. Finally, the optical density (OD) at 570-nm wavelength was measured. The OD value of the nontreated control group was used to indicate 100% cell viability. The cell viabilities (%) of the other groups were calculated by dividing their OD values by the OD value of the nontreated control group.

Caco-2 Intestinal Epithelial Cell Monolayer Preparation

Caco-2 cells were seeded onto permeable 12-well Transwell membranes (Corning, Lowell, MA) with a 3- μ m pore size (cell density of 10⁵/cm²) for 14 d. The culture medium was replaced with fresh medium every 2 d during the culture period. After 14 d, the transepithelial electrical resistance (TEER) was measured using an EVOM Epithelial Voltohmmeter with an STX2 probe (World Precision Instruments, Sarasota, FL). The Caco-2 epithelial monolayers were ready to use when their TEER values were greater than 300 Ω cm² (Zhang et al., 2012).

Measurement of Caco-2 Cell Monolayer Integrity

The Caco-2 monolayers were cocultured with 10⁷ cfu of *Lb. kefiranofaciens* M1 at 37°C for 24 h, which was followed by the addition of 10⁶ cfu of EHEC or EHEC supernatant (20% vol/vol) for 12 h (Johnson-Henry et al., 2008). Transepithelial electric resistance values before coculture with *Lb. kefiranofaciens* M1 and after treatment with EHEC were measured. The TEER change (%) was expressed as the ratio of the TEER after EHEC treatment to the initial value for each experiment group (Zhang et al., 2012).

Statistical Analysis

Values are given as mean $\pm$ standard deviation. All results were analyzed by Student's *t*-test or one-way ANOVA followed by Duncan's multiple range test using SAS software (SAS Institute Inc., Cary, NC). A *P*-value of <0.05 was indicative of a significant difference.

RESULTS

Administration of *Lb. kefiranofaciens* M1 Ameliorates the Symptoms of EHEC Infection

First, we examined the effect of orally administered *Lb. kefiranofaciens* M1 on EHEC O157:H7 challenge using a mouse model. The feed intake per BW was decreased in the EHEC infection group compared with the nontreated control group. Pretreatment with *Lb. kefiranofaciens* M1 was able to prevent the decrease in feed intake caused by EHEC infection (Figure 1A). Administration of *Lb. kefiranofaciens* M1 was also able to improve the symptoms of EHEC infection-induced hemolytic colitis. The fecal occult blood tests showed that 5 out of 9 of the mice (55.56%) in the EHEC infection group had fecal occult blood present, whereas only 1 out of 9 (11.11%) were found to have fecal occult blood present in the *Lb. kefiranofaciens* M1 pretreatment group. Additionally, the increase in cecum weight and the decrease in kidney weight induced by EHEC infection were both restored in the *Lb. kefiranofaciens* M1 pretreatment group; however, no such restoration of the increase in spleen weight was observed (Figure 1B).

Administration of *Lb. kefiranofaciens* M1 Prevents Intestinal Architecture Damage and Reduces Renal Injury Induced by EHEC Infection

The tissue sections of the distal ileum, cecum, and proximal colon were evaluated by histological examination. We observed that the mice pretreated with *Lb. kefiranofaciens* M1 showed a restoration of the atrophy of the microvilli. Furthermore, an amelioration of the loss of crypt and Paneth cells caused by the EHEC infection in distal ileum was also apparent (Figure 2). Enterohemorrhagic *E. coli*-induced tissue edema and loss of epithelial integrity in the cecum (Figure 3A; Supplemental Figure S1, available online at <http://dx.doi.org/10.3168/jds.2013-7015>) and the proximal colon (Figure 3B; Supplemental Figure S2) were also reduced by *Lb. kefiranofaciens* M1 pretreatment. The results were parallel to the qualitative observations outlined above. Pretreatment with *Lb. kefiranofaciens* M1 was found to significantly prevent the cecum and colon tissue damage that was induced by EHEC infection in the mice (Figure 3).

Additionally, severe congestion of the renal glomeruli and kidney interstitial tissue, which was found to be present in the EHEC-infected mice, was found to be largely absent in the *Lb. kefiranofaciens* M1-pretreated rats. Only slight congestion of these tissues (Figure 4) was found, which means that the treatment was able

Figure 1. Effects of administration of *Lactobacillus kefiranofaciens* M1 on feed intake (A) and weight of collected organs (B) of enterohemorrhagic *Escherichia coli* (EHEC)-infected mice. Total feed intake per BW on d 16 was measured and calculated in each experimental group. The cecum, kidney, and spleen of each mouse in different treatment groups were collected and weighed after killing. Means for data without a common letter (a and b) differ significantly ($P < 0.05$). The error bars represent the SD.

to prevent the renal injury induced by EHEC infection in mice. In contrast, moderate congestion was observed in the heat-inactivated *Lb. kefiranofaciens* M1-treated group (Figure 4), which shows that the protective ability of *Lb. kefiranofaciens* M1 was reduced by heat treatment.

Administration of *Lb. kefiranofaciens* M1 Decreases EHEC Translocation and Stx-1/Stx-2 Level in Serum

Infection with EHEC may result in bacterial translocation across intestinal barrier and this can be followed by bacterial migration to the spleen (Figure 5A) and liver (Figure 5B) via the blood stream. Enterohemorrhagic *E. coli* translocation to both spleen and liver was significantly reduced in the *Lb. kefiranofaciens* M1-pretreated group compared with the EHEC-infected group (Figure 5). Surprisingly, no EHEC could be detected in the blood of the *Lb. kefiranofaciens* M1-pretreated group, whereas more than 10^2 cfu/mL of EHEC were found in the blood of the EHEC-infected mice (data not shown).

The levels of the EHEC virulence factors, Stx-1 and Stx-2, in serum and feces across the different treated groups were also examined. The results indicated that the EHEC-infected mice showed significantly higher Stx-1/Stx-2 levels in serum than both the nontreated negative control and the *Lb. kefiranofaciens* M1-pretreated group (Figure 6).

Administration of *Lb. kefiranofaciens* M1 Increases Mucosal EHEC-Specific Immunoglobulin Response

The mucosal and systemic production of immunoglobulin-specific to EHEC O157:H7 were assayed. We observed that the production of EHEC-specific IgA in feces (Figure 7), as well as EHEC-specific IgG and IgM in the serum (data not shown) were all significantly increased in both the EHEC-infected and *Lb. kefiranofaciens* M1-pretreated groups compared with the non-EHEC-treated control. Only the level of EHEC-specific fecal IgA in the *Lb. kefiranofaciens* M1-pretreated group was significantly higher than that of the EHEC-infected positive control (Figure 7). These findings indicate that pretreatment with *Lb. kefiranofaciens* M1 seems to trigger mucosal IgA responses that help to protect against EHEC infection. In contrast, the production of systemic serum total IgA was not increased in the *Lb. kefiranofaciens* M1-pretreated mice compared with the EHEC-infected positive control. The EHEC-infected mice showed a higher serum total IgA concentration than both the noninfected negative control and the *Lb. kefiranofaciens* M1 pretreated groups (data not shown).

Figure 2. Effects of *Lactobacillus kefiranofaciens* M1 on histological evaluation of distal ileum sections of mice infected with enterohemorrhagic *Escherichia coli* (EHEC). Control = nontreated mice; EHEC = EHEC-infected mice; M1 + EHEC = mice pretreated with *Lb. kefiranofaciens* M1 followed by EHEC infection. Color version available in the online PDF.

Putative Mechanisms by Which *Lb. kefiranofaciens* M1 Can Protect Against EHEC In Vitro

The protective effects of *Lb. kefiranofaciens* M1 on intestinal epithelial cells against EHEC-induced damage were investigated in vitro. We found that *Lb. kefiranofaciens* M1 was able to prevent intestinal epithelial cell Caco-2 death caused by EHEC treatment using both an MTT assay (Figure 8A) and an LDH assay (Figure 8B). In addition, the elicited protective effects were found to be significant in a dose-dependent manner when the ratio of *Lb. kefiranofaciens* M1 to EHEC was 10:1 by both MTT assay (Figure 8A) and LDH assay (Figure 8B). This protective effect was not observed when heat-inactivated *Lb. kefiranofaciens* M1 was used (Figures 8A and 8B). Furthermore, when we used a differentiated Caco-2 cell monolayer model to study the protective effects of *Lb. kefiranofaciens* M1, the results showed that *Lb. kefiranofaciens* M1 treatment was able to improve the EHEC-induced loss of Caco-2 monolayer integrity as measured by the TEER value (Figure 8C) and this effect was statistically significant ($P < 0.05$). On the other hand, *Lb. kefiranofaciens* M1 was not able to prevent the damage to the Caco-2 cell monolayer when the bacteria was cocultured with EHEC cell-free supernatant (Figure 9A). Furthermore, *Lb. kefiranofaciens* M1 also failed to reduce Stx levels in the EHEC supernatant (Figure 9B).

DISCUSSION

In the present study, we demonstrated a novel functionality for the kefir-isolated strain *Lb. kefiranofaciens* M1. After pretreatment with *Lb. kefiranofaciens* M1, all symptoms induced by EHEC infection, including fecal occult blood, reduction of feed intake (Figure 1), cecum enlargement, kidney atrophy, ileal villus atrophy (Figure 2), tissue edema, loss of epithelial integrity in the cecum (Figure 3A; Supplemental Figure S1, available online at <http://dx.doi.org/10.3168/jds.2013-7015>), and loss of epithelial integrity in the proximal colon (Figure 3B; Supplemental Figure S2), as well as renal injury (Figure 4), were improved in the mouse model. Similar findings have been reported for other lactobacilli. *Lactobacillus rhamnosus* HN001 (DR20) and *Lactobacillus paracasei* ssp. *paracasei* NTU 101 are able to reduce the severity of EHEC O157:H7 infection (Shu and Gill, 2002; Tsai et al., 2010). *Lactobacillus reuteri* ATCC PTA 6475 is able to ameliorate EHEC infection-mediated complications, such as renal tubular necrosis (Eaton et al., 2011). In terms of bifidobacteria, *Bifidobacterium lactis* HN019, *Bifidobacterium breve* strain Yakult, *Bifidobacterium pseudocatenulatum* DSM 20439, *Bifidobacterium thermacidophilum* RBL 71, *Bi-*

Figure 3. Effects of *Lactobacillus kefirifaciens* M1 on quantitative histological scores of cecum (A) and colon (B) sections of mice infected with enterohemorrhagic *Escherichia coli* (EHEC). Histological scores were evaluated and means for data without a common letter (a–c) differ significantly ($P < 0.05$). Control = nontreated mice; EHEC = EHEC-infected mice; M1 + EHEC = mice pretreated with *Lb. kefirifaciens* M1 followed by EHEC infection. The error bars represent the SD.

fidobacterium longum ssp. *infantis* 157F-4-1, *B. longum* ssp. *longum* NCC2705, and *B. longum* ssp. *longum* JCM 1217^T have also been shown to have the ability to protect against EHEC O157:H7 infection. However, this was the first study to demonstrate that LAB from milk kefir has the anti-EHEC effect.

We further investigated the possible anti-EHEC mechanisms by which *Lb. kefirifaciens* M1 might act in vivo and in vitro. First, we demonstrated that EHEC translocation across the intestinal barrier and the bacterial migration to different organs via the bloodstream was significantly reduced in EHEC-infected mice pretreated with *Lb. kefirifaciens* M1 (Figure 5). In addition,

the concentration of EHEC virulence factors Stx-1 and Stx2 in the serum was also significantly decreased (Figure 6). The reduction in EHEC and Stx translocation after treatment with *Lb. kefirifaciens* M1 might result from an enhancement of mucosal immunity via an elevation of the level of mucosal EHEC-specific IgA secretion in the EHEC-infected mice that had been pretreated with *Lb. kefirifaciens* M1 (Figure 7). A T-cell-dependent high-affinity pathogen-specific IgA response has been reported to play an important role in protection against pathogen infection (Strugnell and Wijburg, 2010). The production of IgA has been found to be associated with activated toll-like receptor 2 (TLR2) and chemokine C-C motif ligand 20 (CCL20). The presence of TLR2 signaling adaptor protein MyD88 [myeloid differentiation primary response gene (88)] is necessary for the production of fecal IgA (Shang et al., 2008). Chemokine CCL20 and its receptor CCR6 pathway are required for localization of dendritic cells to the subepithelial dome of the Peyer's patch (PP); in addition, they have also been found to be responsible for the production of mucosal antigen-specific IgA in both the PP and lamina propria (Cook et al., 2000). In our previous study, *Lb. kefirifaciens* M1 was found in vitro to increase production of chemokine CCL20 in both the apical and basolateral sides of an intestinal epithelial monolayer via TLR2 (Zhang et al., 2012). Both in vitro T helper cell 1 (Th1) cytokine induction and in vivo intestine protection of *Lb. kefirifaciens* M1 have been shown to be dependent on TLR2 (Hong et al., 2009; Zhang et al., 2012). These findings suggest that the anti-EHEC mechanism of *Lb. kefirifaciens* M1 is likely to be related to an increase in mucosal EHEC-specific IgA production via the TLR2/CCL20/CCR6 pathway.

Another possible mechanism by which *Lb. kefirifaciens* M1 might elicit protection against EHEC infection is an enhancement of the functionality of the intestinal epithelial barrier (Figure 8) via TLR2. Toll-like receptor 2 is an important receptor that has been shown to be responsible for protecting the intestinal epithelial barrier against chemicals (Cario et al., 2007) and pathogen-induced colitis (Gibson et al., 2008). Several studies have reported that activation of TLR2 results in (1) enhancement of intestinal epithelial barrier integrity through redistribution of the tight junction protein ZO-1 by the protein kinase C (PKC) pathway (Cario et al., 2004; Gibson et al., 2008); (2) stimulation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, which induces antiapoptosis in intestinal epithelial cells (Cario et al., 2007); and (3) induction of goblet cell-derived trefoil factor (TFF)-3 production (Podolsky et al., 2009). Furthermore, TLR2 signaling adaptor protein MyD88 is also involved in

protection against Stx-mediated disease that is caused by EHEC infection (Calderon Toledo et al., 2008).

Additionally, in our previous study, *Lb. kefiranofaciens* M1 was demonstrated to have an immunity-enhancing ability that occurred by activating macrophages beneath the PP, which, in turn, induced production of Th1 cytokines [IL-1 β , IL-6, IL-12, and tumor necrosis factor α (TNF- α); Hong et al., 2009] via TLR2. Etienne-Mesmin et al. (2011) reported that EHEC is able to translocate into the body through M cells to the PP in mice, where it might be further endocytosed by macrophages. The engulfed EHEC is able to survive in macrophages and this induces macrophage apoptosis in an Stx-2-dependent way, which is followed by release of the Stx, which results in toxicity (Etienne-Mesmin et al., 2011). Therefore, enhancement of the bactericidal activity of macrophages is important to the inhibition of EHEC infection. The bactericidal activity may be enhanced by an increase in the level of cellular hydrogen peroxide via activation of the TLR signaling pathway, including TLR2 (West et al., 2011). Activation of macrophages via TLR2 might be one of the mechanisms by which *Lb. kefiranofaciens* M1 acts against EHEC infection.

Several studies have investigated the possible mechanisms by which lactic acid bacteria elicit protection against EHEC infection. Johnson-Henry et al. (2008) indicated that *Lb. rhamnosus* GG is able to prevent EHEC-induced barrier function loss in vitro by restoring tight junction protein ZO-1 and claudin-1 redistribution, which are induced by EHEC. Putaala et al. (2008) has shown that the barrier function-enhancing strain *B. lactis* 420 is able to block intestinal barrier damage that is induced by Stx secreted by EHEC. *Lactobacillus rhamnosus* HN001 (DR20TM) and *Lb. paracasei* ssp. *paracasei* NTU 101 are able to reduce the severity of EHEC O157:H7 infection by enhancing immune cell function and immunoglobulin production (Shu and Gill, 2002; Tsai et al., 2010). In addition, some studies also reported that certain strains are able to directly eliminate Stx-derived toxicity of intestinal epithelial cells monolayers; this is thought to happen via the binding of Stx by cell lysate, which neutralizes Stx toxicity (Nishikawa et al., 2002). However, we found that *Lb. kefiranofaciens* M1 was not able to remove Stx directly (Figure 9), suggesting that this mode of action is not applicable to *Lb. kefiranofaciens* M1.

Above all, administration of *Lb. kefiranofaciens* M1 was found in this study to be able to prevent or reduce the severity of EHEC infection, which fits the generally accepted concept that inhibition of initial EHEC infection is the best method of preventing EHEC infection and the hemolytic uremic syndrome it mediates (Serna and Boedeker, 2008). Possible mechanisms include the

Figure 4. Effects of *Lactobacillus kefiranofaciens* M1 on histological evaluation of kidney sections of mice infected with enterohemorrhagic *Escherichia coli* (EHEC). EHEC = EHEC-infected mice; M1 + EHEC = mice pretreated with *Lb. kefiranofaciens* M1 followed by EHEC infection; HIM1 + EHEC = mice pretreated with heat-inactivated *Lb. kefiranofaciens* M1 followed by EHEC infection. Color version available in the online PDF.

Figure 5. Administration of *Lactobacillus kefirano-faciens* M1 reduces enterohemorrhagic *Escherichia coli* (EHEC) translocation to the spleen and liver in mice. The EHEC in organs were counted by using CHROMagar O157 (CHROMagar, Paris, France) after homogenization. P -values were calculated by Student's t -test. EHEC = EHEC-infected mice ($n = 4$); M1 + EHEC = mice pretreated with *Lb. kefirano-faciens* M1 followed by EHEC infection ($n = 6$). Color version available in the online PDF.

enhancement of mucosal immunity and improvements in intestinal barrier functionality, both of which will result in the reduction of EHEC translocation to other organs and a decrease in the concentration of EHEC virulence factors in the serum. *Lactobacillus kefirano-faciens* M1 seems to activate macrophages to produce Th1 cytokines and these may also play an important role in pathogen infection defense. Finally, the ability of *Lb. kefirano-faciens* M1 to enhance mucosal immunity is indicative that *Lb. kefirano-faciens* M1 might also be useful as a method of preventing other enteric pathogen infections. These findings might also provide scientific evidence for certain health benefits of milk kefir products.

Figure 6. Administration of *Lactobacillus kefirano-faciens* M1 (M1) decreases serum Shiga toxin-1 and -2 (Stx-1 and Stx-2) levels in enterohemorrhagic *Escherichia coli* (EHEC)-infected mice. Means for data without a common letter (a and b) differ significantly ($P < 0.05$). OD = optical density. The error bars represent the SD.

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Figure 7. Effects of administration with *Lactobacillus kefirano-faciens* M1 (M1) on anti-*Escherichia coli* O157:H7-specific immunoglobulin production in enterohemorrhagic *Escherichia coli* (EHEC)-infected mice. Fecal anti-*E. coli* O157:H7-specific IgA from different treated groups were measured. Means for data without a common letter (a-c) differ significantly ($P < 0.05$). OD = optical density. The error bars represent the SD.

Figure 8. *Lactobacillus kefiranofaciens* M1 (M1) protects intestinal epithelial cell Caco-2 against enterohemorrhagic *Escherichia coli* (EHEC)-induced cell death and monolayer integrity in vitro. Cell viability was evaluated by (A) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and (B) lactose dehydrogenase (LDH) methods. Intestinal epithelial cell monolayer integrity was assayed by measurement of the transepithelial electric resistance (TEER; C). HI-M1 = heat-inactivated *Lb. kefiranofaciens* M1. **P* < 0.05. The error bars represent the SD.

Figure 9. Effects of *Lactobacillus kefiranofaciens* M1 (M1) on enterohemorrhagic *Escherichia coli* cultured supernatant (EHEC-S). (A) Effect of *Lb. kefiranofaciens* M1 on EHEC-S-induced damage to intestinal epithelial monolayer was evaluated by measuring the loss of transepithelial electric resistance (TEER) of the Caco-2 monolayer. The EHEC-S was added 20% (vol/vol) in Caco-2 monolayer cell medium. (B) The effect of *Lb. kefiranofaciens* M1 on the level of Shiga-like toxins (Stx) in EHEC-S was measured by ELISA. OD = optical density. The error bars represent the SD.

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