J. Dairy Sci. 96:7467–7477
http://dx.doi.org/10.3168/jds.2013-7015
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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 foodborne 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

Received May 9, 2013.
Accepted August 31, 2013.
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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 Hemoccult 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

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