Journal of Microbiology, Immunology and Infection (2014) 47, 81—86

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ORIGINAL ARTICLE

Oral administration of *Lactobacillus gasseri* TMC0356 stimulates peritoneal macrophages and attenuates general symptoms caused by enteropathogenic *Escherichia coli* infection

Kazutoyo Yoda, Fang He*, Manabu Kawase, Kenji Miyazawa, Masaru Hiramatsu

Technical Research Laboratory, Takanashi Milk Products Company Limited, Asahi-ku, Yokohama, Kanagawa, Japan

Received 25 June 2012; received in revised form 26 July 2012; accepted 14 August 2012
Available online 15 October 2012

KEYWORDS
Enteropathogenic *E. coli*;
Lactobacillus;
Macrophage;
Phagocytosis

Background: Enteropathogenic *Escherichia coli* (EPEC) is an important cause of diarrhea in human. This study was conducted to investigate the ability of orally administrated probiotic lactobacilli to protect hosts from EPEC infection via enhancement of immune responses.
Methods: Lyophilized *Lactobacillus gasseri* TMC0356 (TMC0356) was orally administered to Institute of Cancer Research (ICR) mice and Sprague Dawley (SD) rats for 11 and 7 days, respectively. These tested mice and rats were intraperitoneally injected with EPEC. Body weight, general symptoms (piloerection, soft stool, diarrhea, and anal hyperemia), and mortality of the tested mice were observed. Peritoneal macrophages were extracted from peritoneal cavity of tested rats, and their phagocytosis and cytokine production were analyzed.
Results: Oral administration of TMC0356 accelerated the disappearance of general symptoms and reduced mortality of EPEC-infected mice in the early phase. Peritoneal macrophages from rats orally administered with TMC0356 showed significant increases in phagocytic activity ($p < 0.05$) and interleukin (IL)-6 production ($p < 0.01$) compared to those from control rats. Tumor necrosis factor-α and production of IL-1β, IL-10, and IL-12 slightly increased, although the changes were not statistically significant.
Conclusion: These results suggest that some of selected probiotic lactobacilli may, at least partly, protect hosts from EPEC infection by the enhancement of innate immunity of host and attenuate symptoms caused by the infection.

* Corresponding author. Technical Research Laboratory, Takanashi Milk Products Company Limited, 5 Honjyuku-cho, Asahi-ku, Yokohama, Kanagawa 241-0023, Japan.
E-mail address: he-fang@takanashi-milk.co.jp (F. He).

1684-1182/$36
http://dx.doi.org/10.1016/j.jmii.2012.08.013

Introduction

Enteropathogenic *Escherichia coli* (EPEC) infection frequently leads to serious disorders such as diarrhea, fever, or death, especially in children and aged persons. Current measures to control the infection rely on the intensive use of antibiotics. However, excessive use of antibiotics has become a major problem because it produces antibiotic-resistant bacteria.[1] In fact, many studies have reported the isolation of antibiotic-resistant EPEC strains from humans.[2,3] In addition, the use of antibacterial agents is often associated with a range of adverse effects, from fever and nausea to major allergic reactions including photodermatitis.[4] For these reasons, alternative therapeutic strategies without antibiotics for enteropathogenic infection should be established.

Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts.[5] Colonization, even temporary, of live cells in the host intestine after oral administration of probiotics is generally considered to be one of the important underlying mechanisms by which probiotics such as lactobacilli and bifidobacteria improve intestinal microbiota and enhance the natural defense of host animals against various infections.[6,7] In several animal studies, some probiotic lactobacilli have shown apparent protective effects against enteropathogens such as *Salmonella typhimurium*, *E. coli*, *Shigella sonnei*, and *Listeria monocytogenes*.[8–10] Therefore, probiotics have been suggested as potent alternatives to antibiotic agents. Mechanisms proposed to account for the protective effects in animal studies include stabilization of the gut mucosal barrier,[11] competition for nutrients, secretion of antimicrobial substances (bacteriocins), and modulation of the mucosal and systemic immune responses.[12] Increasing evidence shows that these protective effects are not genus or species specific, but strain specific.[12,13] Accordingly, additional knowledge about strain-dependent functions and related underlying mechanisms of each probiotics may promote the appropriate use of these helpful bacteria as alternative drugs.

This study was conducted to evaluate the potential protective effects of orally administered *Lactobacillus gasseri* TMC0356 on EPEC infection using both a mouse and a rat model.

Materials and methods

Bacteria

L. gasseri TMC0356 (Takanashi Milk Products Co., Ltd, Yokohama, Japan) was routinely cultured at 37°C for 18 hours in de Man-Rogosa-Sharp broth (Becton, Dickinson, Sparks, MD, USA). After incubation, cultured bacteria collected by centrifugation were washed three times with sterilized saline and lyophilized.

EPEC Juhl strain (supplied by Mercian Corporation, Tokyo, Japan) was grown at 37°C, with shaking in glucose phosphate peptone medium until the mid-exponential phase (absorbance at 660 nm, 1.0), and then diluted with sterilized saline to the viable cell count appropriate for each experiment.

Animal experiments

Male 3-week-old ICR mice and 5-week-old SD rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed at 24 ± 3°C and 55 ± 15% relative humidity with a 12-hour light–dark cycle. The animals were fed a standard diet (MF diet, Oriental Yeast, Tokyo, Japan). All experiments were performed in accordance with the laboratory-animal care guidelines of Mercian Cleantec Co., Ltd (Kanagawa, Japan) and Takanashi Milk Products Co., Ltd.

The mice were divided into three groups—control, *E. coli*, and TMC0356. Lyophilized lactobacilli were orally administered to TMC0356 group mice at a dose of 10 mg/d for 11 days prior to *E. coli* infection and for 1 day after the infection. The other mice were given saline solution orally during the same periods. After 11 days of the experiment, 2×10^7 or 7.5×10^7 cells of *E. coli* were intraperitoneally injected into all test mice except the control mice. Body weight of all mice was measured, and general symptoms (piloerection, soft stool, diarrhea, and anal hyperemia) and mortality were also observed.

The rats were divided into two groups—control and TMC0356—and intraperitoneally injected with 2×10^7 cells of *E. coli* on Day 7 of the experiment. TMC0356 group rats were gavaged with 100 mg of the test bacteria each day throughout the experimental period. Control rats were given saline solution orally during the same period. Two days after infection, rats were sacrificed by exsanguination to obtain peritoneal macrophages without blood contamination. Peritoneal cells were extracted from the peritoneal cavity with 20 mL of ice-cold sterile phosphate-buffered saline (PBS). The extracted cells suspended in RPMI1640 media containing 10% fetal bovine serum (FBS) were plated onto eight-well chamber slides (2×10^5 cells/well) or 24-well culture plates (2×10^6 cells/well), and incubated at 37°C for 1 hour in a 5% CO_2 chamber. After washing by the same media to remove nonadherent cells, the adherent cells were used as peritoneal macrophages.

Macrophage phagocytosis assay

For the phagocytosis assay, latex beads (1.1 μm, Sigma–Aldrich, St Louis, MO, USA) suspended at a concentration of 0.02% in RPMI1640 containing 10% FBS were added to each chamber slide well and incubated with the macrophages at 37°C for 15 minutes. After incubation, the cultures were washed twice with PBS, fixed with methanol, and stained with Giemsa. A total of 300 macrophage cells per rat were counted, the number of macrophages containing one or more latex beads was determined microscopically, and the percent of phagocytic macrophage was defined as the phagocytic rate.

Measurements of cytokines from peritoneal macrophages

Peritoneal macrophages cultured on the 24-well plate were incubated in 900 μL of RPMI1640 containing 10% FBS with 100 μL of lipopolysaccharide (LPS, Sigma–Aldrich) suspension (10 μg/mL in PBS) or PBS, at 37°C for 24 hours, to stimulate cytokine production. Concentrations of interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-

α in the culture supernatant were determined using the sandwich enzyme-linked immunosorbent assay (ELISA) method.

Statistical analysis

The results were expressed as means $\pm$ standard error of the mean (SEM). Statistical comparisons between three groups were made using the Tukey–Kramer test. Differences in the survival rate were examined by Kaplan–Meier method followed by a log-rank test. Comparisons between two groups were calculated by Student *t* test. A *p* value of <0.05 was considered statistically significant.

Results

When 2×10^7 cells of enteropathogenic *E. coli* Juhl were injected into mice, all the mice survived until the end of the experiment (data not shown). The average body weights of the three groups were not significantly different until the day of infection (Day 0: body weight of control, 33.0 ± 0.9 g; *E. coli*, 33.9 ± 0.7 g; and TMC0356, 33.3 ± 0.8 g); however, the body weight of TMC0356 group mice decreased significantly after infection compared to that of control mice (Fig. 1A). *E. coli* group mice did not show any significant differences in average body weight compared with the other group mice during the experimental period except at Day 2. General symptoms including piloerection, soft stool, and diarrhea were observed in most of the infected mice for 2 days after infection (Fig. 1B). The proportion of mice exhibiting these symptoms declined rapidly in the TMC0356 group compared to that in the *E. coli* group during the next 5 days. Irrespective of whether or not TMC0356 was orally administered, the final survival rate of mice infected with 7.5×10^7 cells of *E. coli* dropped to 30% (Fig. 1C).

Phagocytic activity of peritoneal macrophages isolated from infected rats was measured as phagocytic rate (Fig. 2). Oral administration of TMC0356 significantly raised the phagocytic rate in the infected rats.

Concentrations of TNF-α, IL-10, IL-6, IL-1β, and IL-12 in macrophage culture supernatant were measured (Fig. 3). Macrophages isolated from TMC0356 group rats secreted more IL-6 than those from *E. coli* group rats without LPS stimulation ($p < 0.01$). However, no significant differences between *E. coli* and TMC0356 group rats were observed in the production of other cytokines regardless of whether macrophages were stimulated with LPS or not.

Discussion

TMC0356 is a probiotic strain originally isolated from a human intestine.[14] This bacterium as well as its inactive

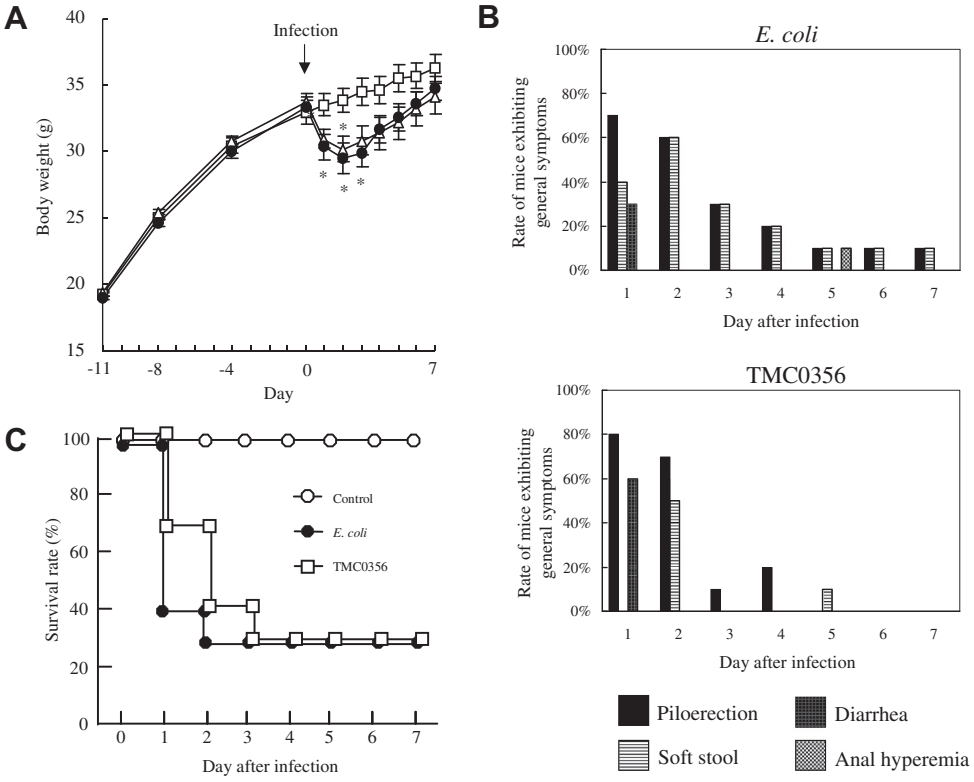

Figure 1. Effects of oral administration of TMC0356 on body weight, general symptoms, and survival rate in mice challenged with pathogenic *E. coli* Juhl. In mice infected with 2×10^7 cells of *E. coli*, transition of body weight during the total experimental period (open squares: control group; open triangles: *E. coli* group; closed circles: TMC0356 group) (A) and rate of mice exhibiting general symptoms including piloerection, soft stool, diarrhea, and anal hyperemia after infection (B). Survival rate (open circles: control group; closed circles: *E. coli* group; open squares: TMC0356 group) of mice challenged with 7.5×10^7 cells of *E. coli* (C). Results of (A) are expressed as means $\pm$ SEM for each group. *$p < 0.05$ compared with control; $n = 10$ in each group (A–C). SEM = standard error of the mean.

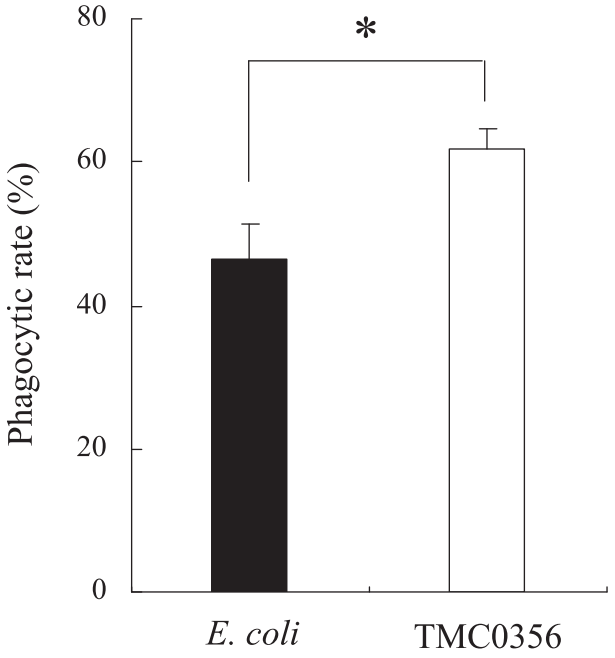

Figure 2. Phagocytic rate of peritoneal macrophages in rats infected with pathogenic *E. coli*. TMC0356 was orally administered to rats for 8 days prior to the infection. After 2 days of the challenge (Day 10), peritoneal macrophages were obtained from the challenged rats to measure the phagocytic activity. Results are expressed as mean ± SEM (*E. coli* group, $n = 8$; TMC0356 group, $n = 7$). *Significant difference from *E. coli* group at $p < 0.05$. SEM = standard error of the mean.

cells can activate macrophages in a strain-dependent manner to induce the production of inflammatory and anti-inflammatory cytokines,[15,16] improve age-associated immune cell-mediated senescence,[17] protect mice from influenza infection,[18–20] and alleviate allergic disorders.[21,22] These results lead to a hypothesis that TMC0356 may protect a host from enteropathogenic infection through its characteristic immune-stimulatory effect, although it does not secrete any antimicrobial agent as do other strains used as probiotics.

In the present study, orally administrated TMC0356 accelerated the disappearance of general symptoms such as piloerection, soft stool, and diarrhea, in mice injected with a low dose of EPEC. The survival rate of TMC0356 group mice challenged with high enteropathogenic bacterial numbers was also elevated for 3 days after infection, although there was no difference between TMC0356 and *E. coli* group mice eventually. These results suggested that TMC0356 may confer protective effects against a mild degree of EPEC infection and attenuate symptoms caused by the infection, especially in the early phase.

Kubota et al reported that oral administration of fermented milk containing *Lactobacillus rhamnosus* GG and TMC0356 stabilized intestinal microbiota and might increase bifidobacteria in patients with Japanese cedar pollinosis.[21,22] Furthermore, the ingested TMC0356 was demonstrated, using strain-specific detection by pulsed-field gel electrophoresis, to survive well and colonize in the host intestine.[23] Based on these results, orally administered TMC0356 was considered to have survived and colonized in the intestine of the tested mice and rats at least temporarily during the intervention of the present study. Activation of TMC0356 in the intestines leading to direct and indirect influence on the intestinal microbiota

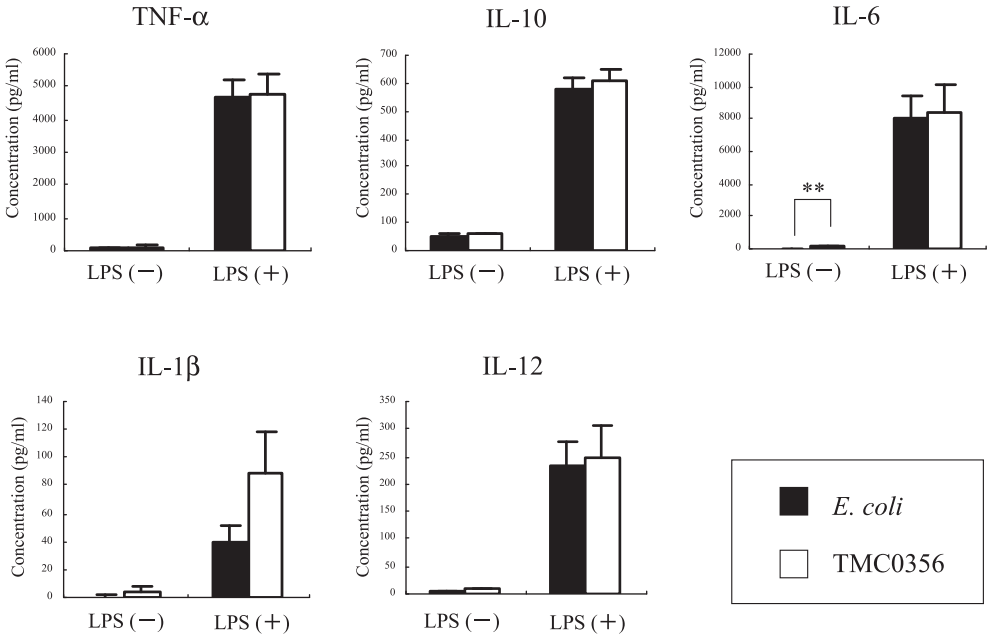

Figure 3. Cytokine production by peritoneal macrophage derived from rats challenged with pathogenic *E. coli*. Peritoneal macrophage obtained from infected rats in each group (*E. coli* group, $n = 8$; TMC0356 group, $n = 7$) were cultured in the absence of LPS, LPS (−), or presence of LPS, LPS (+). Concentrations of TNF-α, IL-10, IL-6, IL-1β, and IL-12 after 24 hours in the supernatant were determined by the sandwich ELISA method. Results are expressed as mean ± SEM. **Significant difference from *E. coli* group at $p < 0.01$. SEM = standard error of the mean.

may be one part of the underlying mechanism behind the preventive effects of TMC0356 on general symptoms caused by EPEC infection. Interestingly, the present study is the first report indicating that TMC0356 administrated alone alleviate defecation problems of host animals, although this was not the main objective of this study.

Macrophages are known as key players of the innate immune system, eliminating pathogens by their phagocytic activity. Contact of macrophages with pathogens provokes the expression of proinflammatory cytokines and mediators that orchestrate pathogen killing and further coordinate the immune response. The present study showed that phagocytic activity of peritoneal macrophage obtained from rats after 2 days of EPEC challenge was markedly enhanced in the TMC0356 group compared to that in the control group. These results suggest that the preventive effects of TMC0356 on EPEC infection may result from the enhancement of the innate immunity of the host by this bacterium, partially with activation of peritoneal macrophages.

In the present study, IL-6 production from isolated macrophages was significantly increased, whereas it was apparently unchanged in the presence of LPS stimulation. These results were in agreement with a previous study in which TMC0356 stimulated the proliferation of mouse spleen cells but did not amplify the proliferation stimulated by mitogens such as concanavalin A.[24] These results are also in good agreements with the previous observation that the immune-stimulatory effects of TMC0356 were mild and different from those of a pathogenic agent such as *E. coli* (data not shown). The mild immune-regulatory effect of TMC0356 may limit the protective effects of this bacterium. However, this characterized immune-regulatory effect of TMC0356 might support the safety of this bacterium for use as a probiotic in human foods.

It is known that a low concentration of IL-6 induces the production of secretory immunoglobulin A (IgA), which is an important factor to protect hosts from pathogens at the mucosal level.[25] However, Harata et al reported that intragastrically administered TMC0356 did not enhance total IgA production by Peyer's patch cells in mice, even though it induced IgA production from isolated murine Peyer's patch cells cocultured with TMC0356 *in vitro*.[26] Therefore, further work is needed to measure total IgA level in a rat model. Based on these findings, the mechanism of the protective effects of TMC0356 administration on pathogen infection may largely be because of the phagocytic activity of macrophages themselves, and not because of the adaptive immunity, involving the production of Th1-cytokines (IL-12 and TNF-α) in macrophages, or humoral immunity. In any case, further work should be conducted to clarify if the actions of TMC0356 on host immunity may be host specific, and know if this bacterium originating from human may behave differently in murine models.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

Acknowledgments

This study was supported by Grant-in-Aid for Research and Development from the Japanese Ministry of Agriculture, Forestry, and Fisheries.

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