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Immunomodulating Activity of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 in Enterohemorrhagic *Escherichia coli* O157H7-Infected Mice

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The present study investigated the immunomodulating activity of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 in enterohemorrhagic *Escherichia coli* O157:H7-infected BALB/c mice. Mice were given *L. paracasei* subsp. *paracasei* NTU 101 (10^8 colony-forming units) for 7 days, before and after the challenge with *E. coli* O157:H7. Feeding *Lactobacillus* for 7 days resulted in an increased postchallenge weight gain and lower cumulative morbidity rates. We observed the upregulation of dendritic cells, helper T cell activation, and antibody production in post- and pretreated mice, compared with untreated mice in the *E. coli* O157:H7 infection group. Moreover, *Lactobacillus* can down-regulate the expression of toll-like receptors (TLRs) on macrophages and proinflammatory cytokines, and chemokines in the post- or prefeeding mice induce by *E. coli* O157:H7 infection. These results demonstrated the inhibition of inflammation among the mice in the pretreated group than in the post-treated group by modulating their immune response. These findings suggest that *L. paracasei* subsp. *paracasei* NTU 101 may be an effective candidate for use as a probiotic in the prevention of infection caused by *E. coli* O157:H7 in humans.

KEYWORDS: ***Escherichia coli*** **O157:H7; probiotics; immunomodulation; Toll-like receptors**

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a highly infectious pathogen that commonly causes gastrointestinal illness in humans, with potentially serious consequences (*1*). EHEC O157:H7 produces Shiga toxins (Stx1, Stx2, or both), lipopolysaccharide (LPS) and hemolysins (*2*−*4*), which could produce diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (HUS), with a mortality rate of approximately 2−10% in humans (*5*). Many cases of infection and outbreak caused by *E. coli* O157:H7 have been reported worldwide. In the USA, multistate outbreaks of *E. coli* O157:H7 infections occurred in 2006 (*6*). In Taiwan, one of 116 (0.86%) specimens of fresh cut vegetables was contaminated with *E. coli* O157:H7 (*7*).

E. coli O157:H7 is considered a worldwide threat because of its increasing incidence and low infectious dose. Colonization of the intestine by pathogenic *E. coli* O157:H7 (*8*) stimulates dendritic cell activation (*9*), inhibition of lymphocyte activation (*10*), and inhibition of antibody production (*11*), and the key virulence factors of Shiga toxins induces intestinal epithelial cell (IEC) cytotoxicity, which enhances the secretion of proinflammatory cytokines and chemokines, such as interleukin (IL)-8 (*12*), binding human platelets through Toll-like receptors (TLR)-4 (*13*). Toll-like receptors are key factors of innate immunity that detect pathogen invasion and trigger responses in the host. Shiga-toxin mediates disease through TLR4 in mice infected with *E. coli* O157:H7 (*14*). TLR5 is an important innate immune receptor, and the interaction of *E. coli* flagellins with TLR5 expressed on the surface of epithelial cells, macrophages, or dendritic cells (DC) initiates a pro-inflammatory cascade of cell signaling (*15*, *16*). Existing evidence does not conclusively support the use of antimicrobial agents to improve the course of illness caused by *E. coli* O157:H7, and it is believed that certain antibiotic treatments may precipitate kidney complications (*17*). Because the overuse of antibiotics has already given rise to antibiotic-resistant strains and with the potential for chronic toxicity, interest is growing in the development of alternatives to control *E. coli* O157:H7 infections, such as the use of probiotics.

Probiotics, including *Lactobacillus*, are defined as live microorganisms providing beneficial effects for the host (*18*). Lactobacilli have shown numerous strain-dependent benefits in the protection of host organisms against a wide variety of enteropathogens, including *Salmonella enterica* serovar Typhimurium (*19*), *Clostridium difficile* (*20*, *21*), *Listeria monocytogenes* (*22*), and pathogenic *E. coli* (*23*). Potential mechanisms to explain the enhanced resistance conferred by antimicrobial lactobacilli include competitive adhesion to intestinal mucosa (*24*), prevention of injury to the

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Table 1. Primer Sequences Used for PCR Analysis[a]

gene	forward	reverse	product size (bp)
IL-1β	5′-GCTGAAAGCTCTCCACCTCAA-3′	5′-GTATTGCTTGGGATCCACACTCT-3′	196 bp
IL-6	5′-GACAACCACGGCCTTCCCTA-3′	5′-GGTACTCCAGAAGACCAGAGGA-3′	302 bp
IL-12	5′-CAGAAGCTAACCATCTCCTGGTTTG-3′	5′-TCCGGAGTAATTTGGTGCTTCACAC-3′	396 bp
IFN-γ	5′-AGCGGCTGACTGAACTCAGATTGTAG-3′	5′-GTCACAGTTTTCAGCTGTATAGGG-3′	243 bp
iNOS	5′-CCTCCTCCACCCTACCAAGT-3′	5′-CACCCAAAGTGCTTCAGTCA-3′	202 bp
MIP-1α	5′-TAGTCACTTTGCGGCTGATG-3′	5′-CAGTGTCAACCCAGGGCTAT-3′	271 bp
MIP-1β	5′-CCCACTTCCTGCTGTTTCTC-3′	5′-GAGGAGGCCTCTCCTGAAGT-3′	238 bp
MCP	5′-ACCAGCCAACTCTCACTGAAGC-3′	5′-CAGAATTGCTTGAGGTGGTTGTG-3′	463 bp
RANTES	5′-CCCTCACCATCATCCTCACT-3′	5′-GGGAAGCGTATACAGGGTCA-3′	297 bp
GAPDH	5′-CCACCTTCTTGATGTCATCA-3′	5′-TATTGGGCGCCTGGTCACCA-3′	752 bp

[a] IL = interleukin; IFN = interferons; iNOS = inducible nitric oxide syntheses; MIP = macrophage inflammatory protein; MCP = monocyte chemotactic protein; RANTES = CCL5 (chemokine ligand 5); GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

epithelial cell barrier (*25*), production of antimicrobial substances that are directly microbicidal for pathogens, and/or immunomodulation of host immune function. However, the mechanisms of immunomodulation are still unknown. It has been demonstrated that the cell wall of *Lactobacillus* contains immunomodulatory components such as polysaccharide and peptidoglycan, which may be influential in activating immune responses (*26*, *27*). It has also been demonstrated that several lactobacilli strains enhance both innate and adaptive immune responses through the induction of antigen presenting cells (APCs), maturation, the proliferation of lymphocytes, modulation of the TLR expression, and further stimulation of cytokines and the production of antibodies (*28*−*30*). Indeed, the inhibition of EHEC infection by lactobacilli has been reported in mouse models (*31*, *32*), but the activity of immune cells is still not understood.

We have previously reported that a human lactobacilli strain, coded *Lactobacillus paracasei* subsp. *paracasei* NTU 101, was isolated from human infant feces and had significantly enhanced innate immunity and induced Peyer's patch-mediated gut mucosal immunity (*33*). The aim of the present study was to investigate the effects of protection, through immunomodulation of *L. paracasei* subsp. *paracasei* NTU 101 against *E. coli* O157:H7 in a murine model.

MATERIALS AND METHODS

Bacteria and Growth Conditions. *L. paracasei* subsp. *paracasei* NTU 101 was inoculated in MRS broth (BD Biosciences, San Jose, CA, USA) and grown under anaerobic conditions using an atmosphere generation system (Oxoid, Basingstoke, Hampshire, England) at 37 °C for 36−48 h. Thereafter, bacteria were resuspended in MRS broth to a final concentration of 1×10^9 CFU (colony-forming unit)/mL. Enterohemorrhagic *E. coli* O157:H7 EDL 933 (ATCC 43895), obtained from Bioresource Collection and Research Center (HsinChu, Taiwan), which produces both Stx1 and Stx2 was grown in Difco tryptic soy broth (TSB; BD Biosciences) at 37 °C for 18−24 h. The number and viability of the lactobacilli and *E. coli* O157:H7 were determined by culturing on MRS and TSA plates for 48 h.

Mice Feeding and Infection Procedure. A murine gastrointestinal infection model was as described previously (*8*, *35*). Specific-pathogen-free (SPF) male (6−8 weeks old) BALB/c mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The mice were divided into 4 groups (8 mice per group). (A) Control group: not given lactobacilli; not challenged. (B) Infected group: not given lactobacilli; challenged with *E. coli* O157:H7. (C) Postinfection feeding group: given 10^8 CFU *L. paracasei* subsp. *paracasei* NTU 101 by oral administration on days 0 through 7; challenged with *E. coli* O157:H7. (D) Preinfection feeding group: given 10^8 CFU *L. paracasei* subsp. *paracasei* NTU 101 by oral administration on days −7 through 0, challenged with *E. coli* O157:H7. Briefly, *E. coli* O157:H7 was suspended at a concentration of 5×10^4 CFU/mL in saline, and a 100-μL portion of the suspension was administered orally to mice. Mitomycin C (MMC; 0.25 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally three times, once each at 18, 21, and 24 h postinoculation. Animal body weight and health appearances were monitored daily. The criteria used for normal and abnormal appearance were in accordance with Shu and Gill (*32*). All data was obtained from three independent experiments. The animal experiments were conducted in accordance with the regulations in the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85-23, revised 1996). Animals were provided with water and Labdiet 5001 chow (PMI Nutrition International, St. Louis, MO, USA) ad libitum.

Immunocytostaining and Flow Cytometry. The mice were sacrificed after *E. coli* O157:H7 infection for 7 days, and the splenocytes were isolated and immunostained in accordance with Tsai et al. (*34*). Immunolabeling was used for the characterization of DC, macrophages, and Th and Tc cell as follows: anti-CD11c-FITC, anti-CD11b-FITC, and anti-CD3-FITC; anti-CD4-PE, anti-I-A/I-E (MHC II)-PE, anti-CD40-PE, and anti-TLR4-PE; antimouse CD80-APC and anti-CD154 (CD40 ligand)-APC; anti-CD86-Cy5 and TLR5-PECy5. The cells were applied to a FACS flow cytometer (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences). The analysis was based on a count of 20,000 cells.

Evaluation of Cytokine Expression by RTPCR. Total RNA from splenocytes was extracted from the pellet using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) to generate cDNA for use in RT-PCR (reverse transcription−polymerase chain reaction). All reactions were performed in a GeneAmp PCR System 2700 (Life Technologies) with the primers as shown in **Table 1**. The PCR products were run on agarose gels and visualized by ethidium bromide staining.

Determination of Antibody Production. Blood samples were collected and centrifuged to separate the sera for antibody assays. IgG, IgM, IgG1, and Ig2a concentrations in serum of BALB/c mice was determined by an indirect enzyme-linked immunosorbent assay (ELISA) with a 1:1000 dilution of horseradish peroxidase-conjugated goat-antimouse IgG, IgM, IgG1, or IgG2a (Zymed, Carlsbad, CA, USA). Optical density (OD) was measured at 405 nm in a PowerWave X340 microplate reader (Bio-Tek, Winooski, VT, USA).

Statistical Analysis. Values shown represent the mean ± SD of separate experiments. Data was analyzed using the one-way ANOVA procedure of SPSS software (SPSS, Inc., Cary, NC, USA). The differences among the means were detected by Duncan's multiple range test. Data was considered significantly different ($p < 0.05$) in variables between groups.

RESULTS

Effect of *L. paracasei* subsp. *paracasei* NTU 101 Feeding on Body Weight and Morbidity. Animal body weight was monitored daily and expressed as an average for each group throughout the experiment. It is noteworthy that no differences in initial mean body weight were detected (day 7 and day 0). Following the challenge with *E. coli* O157:H7, the infected mice showed a significant loss in body weight on day 1 to day 2, particularly in the infected and post-treated with *Lactobacillus* group ($p < 0.05$) (**Figure 1A**). The body weight of mice, in the pretreated group, on day 3 of the week after infection was very similar to that of the mice in the control group. In contrast, the post-treated group did

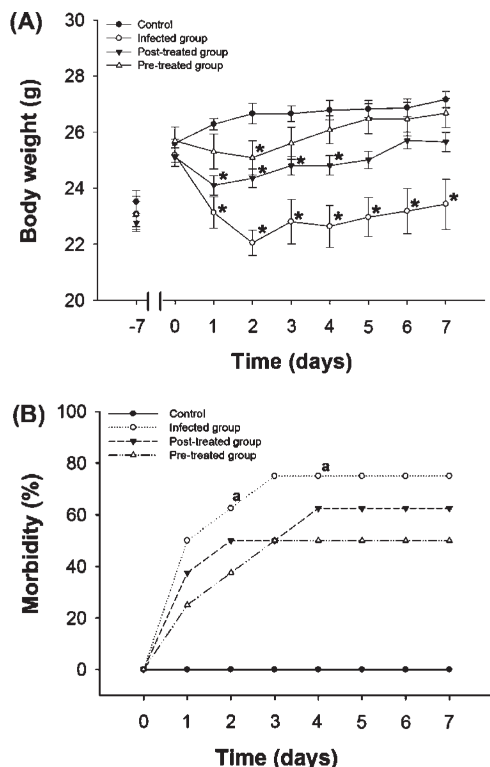

Figure 1. Body weight and cumulative morbidity of *E. coli* O157:H7 challenge in BALB/*c* mice. (**A**) Body weight: all data are presented as the mean ± SE ($n = 8$). * represents significant differences from the control group ($p < 0.05$). (**B**) Morbidity: data express the cumulative percentage of animals with abnormal appearance. Abnormal appearance was expressed as ruffled fur, a loss of sheen of the coat, reduced alertness or activity, and reduced interested in the external environment, signs of hyperventilation when handled, being hunched over or lethargic, being nonreactive to stimuli, agitation, or showing signs of diarrhea. [a] indicates that the animal was dead when observed.

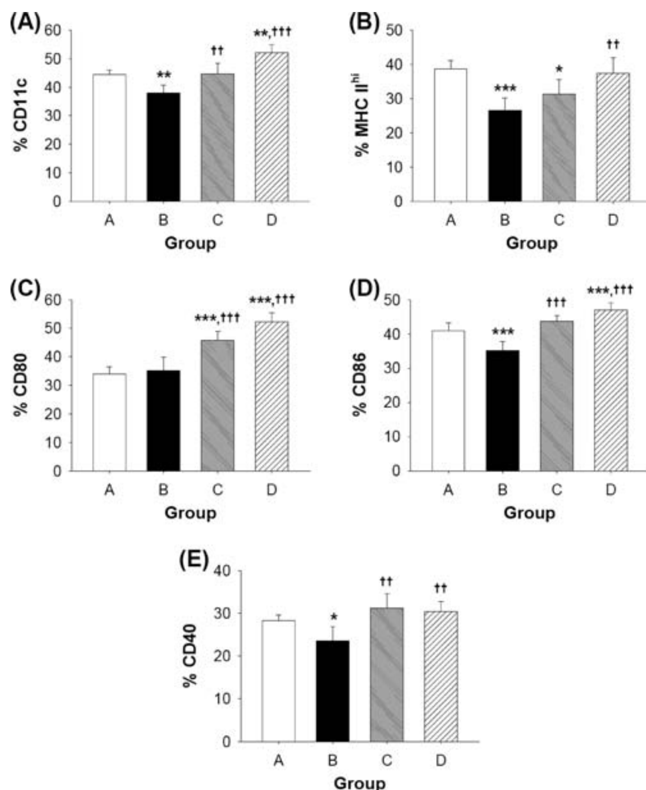

Figure 2. *L. paracasei* subsp. *paracasei* NTU 101 upregulates the maturation of DC surface marker expression after *E. coli* O157:H7 infection. Upon isolating splenocytes from mice, cells were examined by flow cytometry after oral administration of *L. paracasei* subsp. *paracasei* NTU 101. Group A: not given *L. paracasei* subsp. *paracasei* NTU 101 (NTU 101); not challenged. Group B: not given NTU 101; challenged with pathogen. Group C: given NTU 101 on days 0 through day 7; challenged with pathogen. Group D: given NTU 101 on days −7 through day 7; challenged with pathogen. Results of (**A**) CD11c; (**B**) MHCII^{hi}; (**C**) CD80; (**D**) CD86; and (**E**) CD40 expression on DCs are shown. All data are presented as the mean ± SD ($n = 8$). *, **, and *** represent significant difference from the control group ($p < 0.05$, 0.01, and 0.001). †† and ††† represent significant difference from the infected group ($p < 0.01$ and 0.001).

not maintain a normal body weight curve on day 5 postinfection. The results in appearance following infection with *E. coli* O157:H7 mice showed lower cumulative morbidity rates in pre- and postfeeding with *L. paracasei* subsp. *paracasei* NTU 101. Moreover, one of the mice that died was observed on day 2 and day 4 after *E. coli* O157:H7-challenge in the infected group but not in the post- or pretreated groups (**Figure 1B**). Furthermore, splenomegaly accompanied with semisolid stool in the colon was observed in moribund mice. Therefore, all regimens of *Lactobacillus* feeding had prevented weight loss and morbidity caused by the *E. coli* O157:H7 challenge, particularly in the pretreated mice.

***L. paracasei* subsp. *paracasei* NTU 101 Upregulate the Maturation of DC Surface Marker Expression after *E. coli* O157:H7 Infection.** To investigate surface marker expression on DCs at pre- or postinfection of *E. coli* O157:H7, flow cytometry analysis was performed. The data showed that activation of CD11c, MHC class II^{hi}, CD40, and CD86 was significantly decreased in the control group after *E. coli* O157:H7 infection ($p < 0.05$). In the post-treated group, the expression of MHC II^{hi} had been downregulated compared with that in control group ($p < 0.05$), but the level of decrease showed slight moderation when compared with the infected group. Moreover, the effect of costimulatory molecular expression had significantly increased from that of the infected group after the administration of *Lactobacillus* for 7 days ($p < 0.05$) (**Figure 2**). Similar but more marked trends were found when analyzing specifically for cell surface markers on DCs in the pretreated group. *Lactobacillus* modulated the DCs phenotype by upregulating MHC II^{hi}, activating costimulatory molecules CD40, CD80, and CD86 after infection, even more than in the control group (**Figure 2C** and **D**) ($p < 0.001$). These results indicated that *E. coli* O157:H7 could reduce the antigen presenting ability of DCs and that the impairment of the surface marker replies normally with pretreatment of *L. paracasei* subsp. *paracasei* NTU 101. Moreover, the impairment level of DC maturation showed greater regulation in the group pretreated with *Lactobacillus*.

***L. paracasei* subsp. *paracasei* NTU 101-Induced CD154 Molecule Expression of Spleen Lymphocytes after *E. coli* O157:H7 Infection.** As indicated in our previous experiments, DC activation was replaced by *L. paracasei* subsp. *paracasei* NTU 101 after *E. coli* O157:H7 infection. On the basis of the knowledge that DCs activate an adaptive immune response to produce cytokine and antibodies via interaction with a helper T cell, we examined the influence of CD154 expression on splenic CD4^{+} T cells. As shown in **Figure 3A**, the percentage of CD4^{+} T cells in the *E. coli* O157:H7-treated mice had significantly increased ($p < 0.05$), but there was no effect on CD4^{+} T cells either post- and pre-oral administration of *Lactobacillus* compared with that in the the infected group. In splenocytes from *E. coli* O157:H7-treated mice,

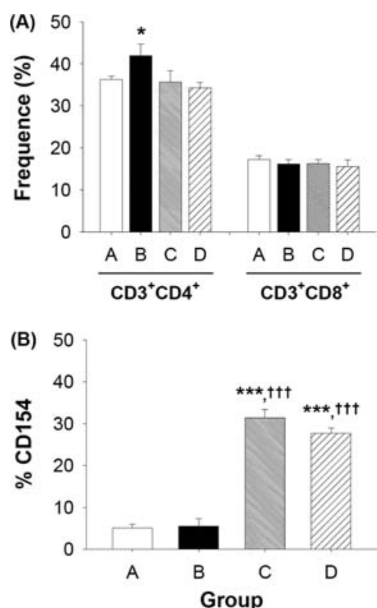

Figure 3. *L. paracasei* subsp. *paracasei* NTU 101-induced CD154 molecule expression of spleen helper T cell after *E. coli* O157:H7 infection. Upon isolating splenocytes from mice, cells were examined by flow cytometry after infection or oral administration with *L. paracasei* subsp. *paracasei* NTU 101. Group A: not given NTU 101; not challenged. Group B: not given NTU 101; challenged with pathogen. Group C: given NTU 101 on days 0 through day 7; challenged with pathogen. Group D: given NTU 101 on days −7 through day 7; challenged with pathogen. Results of (**A**) $CD4^+$ and $CD8^+$ and (**B**) CD154 expression on helper T cells are shown. All data are presented as the mean $\pm$ SD (n = 8). * and *** represent significantly different results from those of the control group ($p < 0.05$, and 0.001). ††† represents significantly different results from those of the infected group ($p < 0.001$).

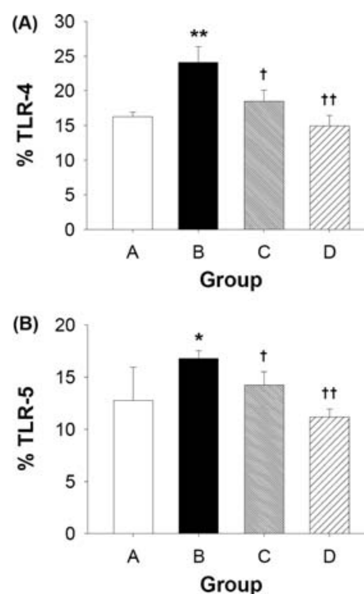

Figure 4. Reduction of TLR4 and TLR5 overexpression by *L. paracasei* subsp. *paracasei* NTU 101 after *E. coli* O157:H7 infection. Upon isolating splenocytes from mice, cells were examined by flow cytometry after infection or oral administration with *L. paracasei* subsp. *paracasei* NTU 101. Group A: not given NTU 101; not challenged. Group B: not given NTU 101; challenged with pathogen. Group C: given NTU 101 on days 0 through day 7; challenged with pathogen. Group D: given NTU 101 on days −7 through day 7; challenged with pathogen. Results of (**A**) TLR4 and (**B**) TLR5 expression on macrophage are shown. All data are presented as the mean $\pm$ SD (n = 8). * and ** represent significantly different results from those of the control group ($p < 0.05$, and 0.01). † and †† represent significantly different results from those of the infected group ($p < 0.05$ and 0.01).

no effect on the percentage of $CD8^+$ T cells was apparent, even after treatment with lactobacilli. **Figure 3B** shows that the activation of CD154 on $CD4^+$ T cells of the post- or pretreated groups increased to 31.4% and 27.7%, which was 5.7- and 5.0-fold higher than in the infected group ($p < 0.05$). This data indicates that *L. paracasei* subsp. *paracasei* NTU 101 had upregulated the CD154 expression on $CD4^+$ T cells after *E. coli* O157:H7 infection.

Reduction of TLR4 and TLR5 Overexpression by *L. paracasei* subsp. *paracasei* NTU 101. In general, TLR4 or TLR5 on macrophages is able to recognize lipopolysaccharide and bacterial flagellin and accompany the activation of a signaling cascade leading to a variety of host immune responses to the pathogen. We determine whether a *Lactobacillus* would be able to influence the TLR4 or TLR5 expression on macrophages via *E. coli* O157:H7 infection. As seen in **Figure 4**, TLR4 and TLR5 expressions increased, mediated by *E. coli* O157:H7 infected mice ($p < 0.01$ and 0.05). But the level of activation was significantly decreased in both post- and pre-oral administration of *Lactobacillus* compared with that in the infected group ($p < 0.05$ and 0.01). Moreover, the activation of TLR4 and TLR5 was shown to be greater regulated in pretreated with the *Lactobacillus* group than in the post-treated group. These observations indicated that *L. paracasei* subsp. *paracasei* NTU 101 could down-regulate TLR expression via induction by *E. coli* O157:H7 infection with an unknown mechanism.

Effect of Cytokine and Chemokines Expression in *E. coli* O157:H7 and *L. paracasei* subsp. *paracasei* NTU 101. To investigate the effects of immunomodulation of *E. coli* O157:H7 infected mice, the cytokine and chemokine mRNA expression levels of the mice of the four groups were observed and compared. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is expressed at relatively constant levels in cells. The results showed a reduction in pro-inflammatory cytokines, including IFN-γ and IL-12 after treatment with *E. coli* O157:H7. However, there were large amounts of IFN-γ and IL-12 mRNA, following post- or pretreatment with *Lactobacillus*. The level of inflammatory cytokines IL-1β, IL-6, and iNOS gene expression was somewhat stronger in the infected group compared with that in the mice that had been post- or pretreated with *Lactobacillus*; however, the data showed no significant change in iNOS expression in either the post- or the pretreated group (**Figure 5A**). Similar trends for MIP-1α, MIP-1β, and MCP chemokines were observed after treatment with *E. coli* O157:H7 and *Lactobacillus*, but the data showed no significant change in the expression of RANTES mRNA (**Figure 5B**). These results suggested that splenocytes had been induced toward a Th1 response and a down-regulation of inflammatory expressions after the mice were treated with *L. paracasei* subsp. *paracasei* NTU 101.

Effect of Antibody Concentration after Treatment with *L. paracasei* subsp. *paracasei* NTU 101 or *E. coli* O157:H7. After infecting mice with *E. coli* O157:H7, we observed a significant decrease in IgG antibodies in serum compared with that in control mice ($p < 0.05$) but an increase in the concentration of IgG antibodies in the group post- or pretreated with *Lactobacillus* ($p < 0.01$ and 0.001; **Figure 6A**). Moreover, the IgG concentration was shown to be higher in the pretreated group ($p < 0.01$) than in the control mice. Similar to the results shown in **Figure 6B**, the IgM antibodies increased in post- and pretreated mice

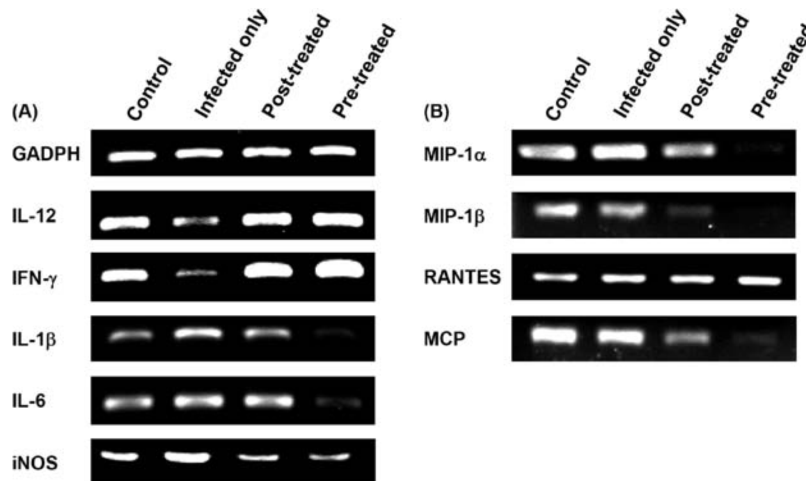

Figure 5. Effect of *L. paracasei* subsp. *paracasei* NTU 101 on cytokine and chemokine expression in the spleen after *E. coli* O157:H7 infection. Upon isolating splenocytes from mice, cells were examined by RTPCR after infection or oral administration with *L. paracasei* subsp. *paracasei* NTU 101. Control group: not given NTU 101; not challenged. Infected group: not given NTU 101; challenged with pathogen. Post-treated group: given NTU 101 on days 0 through day 7; challenged with pathogen. Pretreated group: given NTU 101 on days −7 through day 7; challenged with pathogen. The data shown are representative of typical results.

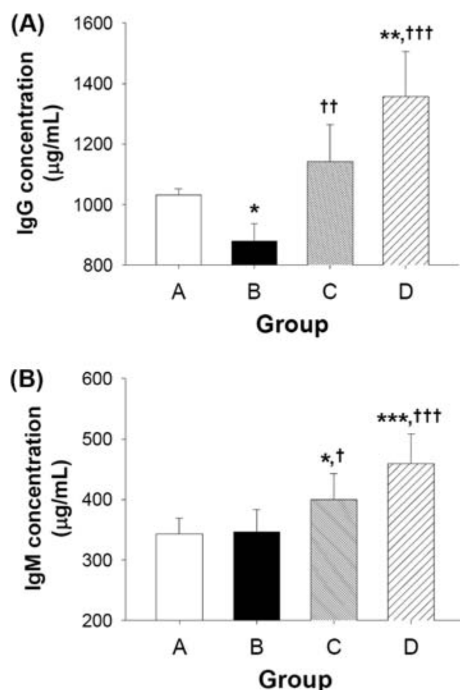

Figure 6. *L. paracasei* subsp. *paracasei* NTU 101-induced serum antibodies secreted after *E. coli* O157:H7 infection. Upon isolating splenocytes from mice, the serum antibody was examined by ELISA after infection or oral administration with *L. paracasei* subsp. *paracasei* NTU 101. Group A: not given NTU 101; not challenged. Group B: not given NTU 101; challenged with pathogen. Group C: given NTU 101 on days 0 through day 7; challenged with pathogen. Group D: given NTU 101 on days −7 through day 7; challenged with pathogen. Results of serum (**A**) IgG and (**B**) IgM concentration. All data are presented as the mean ± SD (n = 8). *, **, and *** represent significantly different results from those of the control group ($p < 0.05$, 0.01, and 0.001). †, ††, and ††† represent significantly different results from those of the infected group ($p < 0.001$).

compared with the control and infected groups ($p < 0.05$ and 0.001). For IgG subclasses, a decrease in IgG1 and IgG2a OD values was measured from 7 days after *E. coli* O157:H7 infection ($p < 0.05$ and 0.01), with no significant change in IgG1 and

Table 2. Titers of Serum IgG Subclass Antibodies on BALB/*c* Mice after *E. coli* O157:H7 Infection or Oral Administration of *L. paracasei* subsp. *paracasei* NTU 101[a]

IgG subclass	group/O.D. value			
	A	B	C	D
IgG2a	1.25 ± 0.11	1.01 ± 0.04*	1.19 ± 0.16	1.39 ± 0.10
IgG1	0.90 ± 0.07	0.78 ± 0.03*	0.84 ± 0.05	0.88 ± 0.04
IgG2a/IgG1 ratio	1.39	1.29	1.41	1.57

[a] All data are presented as the mean ± SD (n = 8). *, represents significant difference from the control group ($p < 0.05$).

IgG2a antibody production in the groups post- or pretreated with *Lactobacillus*, when compared to the control mice (**Table 2**). A similar trend was shown in the ratio of IgG1 to IgG2a. These results indicated that treatment with *Lactobacillus* could upregulate the secretion of IgG and IgM and reduce the effect of the IgG subclass, especially in the group pretreated with *L. paracasei* subsp. *paracasei* NTU 101.

DISCUSSION

Previous research has confirmed that *Lactobacillus* inhibits Stx toxins by triggering adaptive immune responses. This protects the host and alleviates symptoms caused by *E. coli* O157:H7 infection (*36*). However, the immune mechanism of *Lactobacillus* in regulating pathogen infection remains unknown. In this study, mice were infected with *E. coli* O157:H7, which induced intestinal bleeding. They were fed *L. paracasei* subsp. *paracasei* NTU 101, and the protective effect of *Lactobacillus* was evaluated, on the basis of the innate and adaptive immunoregulatory abilities of the spleen. This study aimed to gain insight into the immunomodulation mechanisms against pathogen infection after the administration of *Lactobacillus*.

During the period of the experiment, body weight and morbidity of all mice were evaluated. The average body weight of the infected group was continuously lower than those of other groups throughout the experiment, and animal death occurred on the second and fourth days after infection. As for the post-treated and pretreated groups, body weight returned to normal five and three days postinfection, respectively. Morbidity of the mice showed signs of decreasing. This indicated that the feeding

of *Lactobacillus* had effectively alleviated the physiological effects (such as weight loss) induced by *E. coli* O157:H7 infection. It is worth noting that a period of *Lactobacillus* feeding prior to *E. coli* O157:H7 infection provided better protection. This improvement can be observed in the pretreated group, as the body weight quickly returned to normal as the morbidity decreased.

Results from assays of immune-related activity indicated that the antigen presenting abilities of dendritic cells had reduced after infection with *E. coli* O157:H7. Previous research confirmed that *K. pneumonia* of the genus *Enterobacter* in the family Enterobacteriaceae had increased stimulatory activities on the surface of antigen presenting cells through the induction of OmpA (*37*). However, *E. coli* O157:H7, also of the genus *Enterobacter* in the family Enterobacteriaceae, in this study suggests that different bacteria reacted differently to immunosuppression. This revealed that different bacterial strains reacted differently to the antigen presenting abilities of dendritic cells. Feeding *L. paracasei* subsp. *paracasei* NTU 101 postinfection significantly alleviated the decrease of antigen presenting activity. On the contrary, feeding of *L. paracasei* subsp. *paracasei* NTU 101 for 7 days preinfection resulted in an increase rather than a decrease in overall antigen-presenting activity. Previous research showed that *L. paracasei* subsp. *paracasei* NTU 101 effectively enhanced the activity of antigen presenting cells (*34*). From the results of this study, it can be concluded that administration of *Lactobacillus* amended the immunomodulation activity induced by *E. coli* O157:H7 infection. Preinfection administration of *Lactobacillus* led to even better results for protection. Activated $CD4^+$ T cells were the predominant cell type expressing CD154 (CD40 ligand) and were pivotal in cell-to-cell communication (*38*). Our previous research had shown that *L. paracasei* subsp. *paracasei* NTU 101 enhanced the CD154 activity of helper T cells and induced the interaction of CD40 with dendritic cells (*33*). Therefore, this study investigated the effect of *E. coli* O157:H7 infection on helper T cells. Results showed that in both the pretreated and post-treated with *Lactobacillus* groups, the activity of helper T cells had been enhanced. This was a clear indication that the host's immune system could be activated through the administration of *L. paracasei* subsp. *paracasei* NTU 101 even after *E. coli* O157:H7 infection, thereby providing protection through immunity.

In this study, we observed cytokine expressions in the spleen by RT-PCR and revealed that the expression of IFN-γ and IL-12 had decreased after infection with *E. coli* O157:H7. Pretreated and post-treated groups however, displayed IFN-γ and IL-12 expression levels similar to those of the control group, which was significantly higher than that of the infected group. Interaction between dendritic cells and CD154 of helper T cells induced high-level expressions of Th1 cytokine IL-12, and *Lactobacillus* had induced the secretion of cytokine IL-12 in dendritic cells, which triggered immune responses toward the T helper type 1 polarizing program (*39*−*41*). Our previous study indicated that *L. paracasei* subsp. *paracasei* NTU 101 enhanced IFN-γ and IL-12 expression in the spleen and Peyer's patch of the intestine (*33*). This indicated that *L. paracasei* subsp. *paracasei* NTU 101 possesses the ability to recover cell activity and cytokine expression losses after infection with *E. coli* O157:H7. In adaptive mucosal immune response to postinfection of *E. coli* O157:H7, antibody expression served as a critical protection factor (*42*). Previous research confirmed that antigen recognition to flagella induced specific IgG antibody secretion, which interfered with TLR5 overexpression caused by the flagella of *E. coli* O157:H7 (*43*). For this reason, this study investigated serum antibody levels. Results showed that IgG, IgG2a, and IgG1 antibody levels in the serum had decreased after *E. coli* O157:H7 infection, while pretreated and post-treated groups displayed higher IgG antibody secretion levels than those in the infected group. Furthermore, the pretreated group exceeded group A in IgG antibody secretion. These results indicated that after infection with *E. coli* O157:H7, antibody secretion was affected due to a decrease in immunocyte activity. Administration of *Lactobacillus*, however, enhanced helper T cell activity and cytokine expression, which in turn induced antibody production and led to immunity for the host.

Stx toxin of *E. coli* O157:H7 is cytotoxic to intestinal epithelial cells and causes secretion of chemokines IL-1β, IL-6, and iNOS (inducible nitric oxide syntheses) as well as tumor necrosis factor (TNF)-α (*44*, *45*). These factors, involved in the acute inflammatory response, could increase vascular permeability and attract activated lymphocytes and macrophages. Previous research showed that *E. coli* K4 enhanced the expression of proinflammatory cytokines such as IL-1β and IL-8 (*46*). Investigation of proinflammatory cytokine and chemokine expression in this study showed that chemokine RANTES (CCL5) expression of T cells was not affected by infection or the administration of *Lactobacillus*. This was consistent with the results of previous research (*47*). The expression of inflammation-related factors IL-1β, IL-6, iNOS, MIP-1α, MIP-1β, and MCP increased after infection with *E. coli* O157:H7, indicating that *E. coli* O157:H7 infection had led to strong inflammatory responses in the host. However, expression of inflammation-related factors was lowered by the administration of *Lactobacillus*, indicating that *L. paracasei* subsp. *paracasei* NTU 101 had alleviated postinfection inflammatory responses. This inhibition was greater in pretreated groups than in post-treated groups. The above results led to the conclusion that a decrease in immunocyte activity and enhancement of inflammation-related factor expression levels after infection with *E. coli* O157:H7 had all been alleviated by the administration of *L. paracasei* subsp. *paracasei* NTU 101.

TLR4 and TLR5 on macrophages mediate cellular activation in response to LPS and flagellin derived from either *E. coli* and *Salmonella Minnesota*, and activation of the TLRs receptor mobilizes the nuclear factor NF-$\kappa\beta$ and mitogen-activated protein kinase (MAPK) family members, resulting in a stimulation of cytokine and chemokine production in the macrophages of mice (*48*, *49*). In *Salmonella enterica* serovar Typhimurium or *Streptococcus pyogenes*, treatment of different *Lactobacillus* strains led to a variety of regulatory effects in TLRs (*35*, *50*), and this study, therefore, evaluated the activity of TLR4 and TLR5 receptors. Results showed that the infected group displayed a significant increase in TLR4 and TLR5 expression levels, indicating that the toxin-induced postinfection group had affected the host via TLR receptors on macrophages. However, in *Lactobacillus*-administrated groups, TLR4 and TLR5 expression levels were significantly lower than those in the infected group. Expression levels decreased to levels similar to those in the control group. Previous research showed that *Lactobacillus suntoryeus* had inhibited NF-$\kappa\beta$ activity induced by TLR4 in mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced intestinal irritation (*51*). This indicated that *L. paracasei* subsp. *paracasei* NTU 101 could alleviate infection by blocking *E. coli* O157:H7 toxins from attacking TLRs in host immunocytes.

In conclusion, our data suggest that *L. paracasei* subsp. *paracasei* NTU 101 could modulate antigen presentation, helper T cell activation, antibody production, and inhibit inflammation by regulating the pro-inflammatory cytokines and down-regulating the expression of TLRs on macrophages to reduce the effects of *E. coli* O157:H7 infection. Treatment of BALB/c mice with *Lactobacillus* reduced the severity of *E. coli* O157:H7 infection in vivo. Moreover, the level of damage was greater in the group pretreated with *Lactobacillus* than in the post-treated group. This study suggested the therapeutic potential of *L. paracasei* subsp.

paracasei NTU 101 in the treatment and even more so in the prevention of diseases such as enterohemorrhagic *E. coli* infections. Supplementation of human diets with *L. paracasei* subsp. *paracasei* NTU 101 could provide health benefits.

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