보건학석사학위논문

# Screening and evaluation of *Lactobacillus* spp. tightening the intestinal barrier

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2017 년 8 월

서울대학교 보건대학원

환경보건학과 환경보건학 전공

**MEINA LIN**

# Abstract

## Screening and evaluation of *Lactobacillus* spp. tightening the intestinal barrier

**Meina Lin**

**Dept. of Environmental Health Sciences**

**The Graduate School of Public Health**

**Seoul National University**

The intestinal barrier plays an important role in the interactions between host and microbiome for nutrient absorption and immune regulation while also acting as the physical barrier preventing the transport of a variety of harmful substances from the gut to the bloodstream. *Lactobacillus* spp. has been researched a lot as probiotics for an alternative treatment in inflammatory gut diseases, while further sustainable evidence of their beneficial effects is required. This study

aimed to 1) screen probiotic candidates of *Lactobacillus* spp. isolated from Korean feces using a transepithelial/transendothelial electrical resistance (TEER) assay, 2) evaluate their ability on tight junction proteins ZO-1 and Occludin, 3) find if supernatant or heat-killed bacteria sustained the beneficial effects as live probiotics. A TEER assay was conducted with 21 strains of *Lactobacillus* spp. to screen probiotic candidates and species of *L. rhamnosus* were shown to increase relative TEER change significantly in comparison to the control. We selected 3 strains of *Lactobacillus* spp. (KBL363, KBL365 and KBL385) which demonstrated the most increasing effects in TEER change. Repeated TEER assay was conducted to investigate sustainable beneficial effects on the intestinal barrier and the assay results showed that probiotic candidates sustained beneficial effects for 24 h, with the most significant tightening effects seen in the first 12 h period. In accordance to TEER results, 3 selected probiotic strains significantly increased tight junction proteins ZO-1 and Occludin. We further investigated the effects of bacteria culture supernatant and heat-killed bacteria on intestinal barrier functionality using the 3 selected probiotic strains. However, unlike other studies describing beneficial effects of culture supernatant, there was no significant up regulation in TEER change observed in response to

treatment with culture supernatant. In contrast to supernatant treatment, heat-killed probiotic candidates increased intestinal barrier functions in TEER assay and also enhanced tight junction proteins significantly, indicating that outer membrane vesicles may play a role in the tightening effects, although clear mechanisms are not yet understood. In conclusion, this study determined 3 strains of *Lactobacillus* spp. with probiotic potential to increase intestinal barrier functionality and also provided a mechanical suggestion underlying which bacterial components are responsible for tightening effects in intestinal barrier.

Key words: intestinal barrier, *Lactobacillus* spp., heat-killed probiotics, probiotic supernatant.

**Student No. 2015-24069**

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# I. Introduction

The intestinal barrier is the layer of epithelial cells lining the gut wall that exhibits some permeability for absorbing nutrients, electrolytes, and water while constituting the critical barrier against antigens, pathogens and harmful substances [1]. The intestinal barrier is regulated by cell-cell junctions called tight junctions, of which the major proteins are Zonula occludens-1 (ZO-1), Occludin and Claudins [2-4]. The barrier can provide strong defense due to tight junctions effectively sealing the cell-cell paracellular space [5]. Maintaining the intestinal barrier 'tightly' is essential for human health, and a 'leaky' intestinal barrier is a key factor in the development of several metabolic diseases such as obesity, diabetes and gut dysfunctions including irritable bowel syndrome, Crohn's disease and ulcerative colitis [6-10].

Microbiota is the link between intestinal barrier and human health, as shown in the fact that selective gut microbiota changes improve gut barrier functions [11-13]. Probiotics are living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition [14]. There is increasing evidence that probiotic bacteria, most notably the *Lactobacillus* genera

whose safety and stability have been validated thoroughly, collected from healthy people have been effective in the prevention and treatment of metabolic diseases and gastrointestinal inflammatory disease [15-17]. There are clinical trials that confirm evidence for the use of probiotics in irritable bowel syndrome, Crohn's disease and ulcerative colitis [18].

Probiotics-induced strengthening of the barrier results in intestinal epithelial homeostasis, regulation of immune function and improvement of metabolic effects [19]. However, the mechanisms underlying these healthy effects are not yet fully understood. Probiotics interact with the host through various cell signaling and receptor interactions. Cani et al. (2009) illustrated that improvement in intestinal barrier is associated with a mechanism involving the glucagon-like peptide-2 [11]. Karczewski et al. (2010) also demonstrated that probiotics stimulate Toll Like Receptors (TLRs), such as TLR2, to regulate intestinal barrier functionality as a part of an investigation of the mechanisms involved in cell signaling [1]. However, there are fewer studies about probiotic components than there are studies describing intracellular mechanisms that trigger beneficial effects. Secreted bioactive factors from probiotic showed enhancement in intestinal barrier functions [20].

Although there are several probiotic strains have been researched,

exploration of probiotic candidates tightening intestinal barrier is required and the sustainable effects should be confirmed with repeated experiments. In this study, the effects of 21 strains of *Lactobacillus* spp. isolated from Korean feces on intestinal barrier were evaluated using TEER assay and probiotic candidates were screened out with three individual repeated experiments. To investigate which bacterial components were responsible for tightening effects in intestinal barrier, bacterial culture supernatant and heat-killed bacteria were tested as well.

## II. Materials and Methods

### 1. Cell culture

Human Caco-2 cells which form a confluent monolayer have been the typical *in vitro* model of intestinal barrier function studies [21]. Caco-2 cell line was obtained from American Type Culture Collection and stored in a liquid nitrogen tank. Caco-2 cells were maintained in Minimum Essential Media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; GenDEPOT, Barker, TX, USA), 1% non-essential amino acids solution (Thermo Fisher Scientific, Waltham, MA, USA), 1% HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 1.5% sodium bicarbonate solution (Thermo Fisher Scientific, Waltham, MA, USA), 50 μg/ml gentamicin (Thermo Fisher Scientific, Waltham, MA, USA) and 10 U/ml penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and incubated in a humidified incubator at 37°C with 5% $CO_2$. Passage 30-40 cells were used for this experiment and cells were subcultured every 3-4 days by trypsin (Thermo Fisher Scientific, Waltham, MA, USA).

## 2. Bacterial strains and culture conditions

Twenty-one strains of nine species in *Lactobacillus* spp. were isolated from fecal samples of healthy adults and infant feces. *Escherichia coli* was used as a negative control and isolated from infant feces (data not published). All isolates were identified to the species level by sequencing of 16S rRNA and EzBioCloud's Identify Service (http://www.ezbiocloud.net) (Table 1). These isolates were selected on the basis of their resistance to bile salt and low pH (data not published). *Lactobacillus* spp. were cultivated in MRS medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.05% L-cysteine-hydrochloride (Sigma-Aldrich, St Louis, MO, USA) at 37°C in anaerobic conditions for 24 h. Dead bacteria were cultured as the same conditions as live bacteria followed by heat killing at 70°C for 30 min. Bacterial supernatant was gathered by centrifugation (Eppendorf, Hamburg, Germany) at 16000g for 5 min followed by filtration. *E. coli* were cultured in LB broth (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 12 h.

**Table 1. Bacterial sample list.**

| Genus | Species | Strain | Isolated from |
|---|---|---|---|
| *Escherichia* | *Escherichia coli* | *E. coli* O157 EC4115 | Infant feces |
| *Lactobacillus* | *Lactobacillus gasseri* | KBL342 | Infant feces |
| | | KBL381 | Adult feces |
| | *Lactobacillus reuteri* | KBL346 | Infant feces |
| | *Lactobacillus rhamnosus* | KBL351 | Infant feces |
| | | KBL354 | |
| | | KBL362 | |
| | | KBL363 | |
| | | KBL365 | |
| | *Lactobacillus fermentum* | KBL374 | Adult feces |
| | | KBL375 | |
| | *Lactobacillus casei* | KBL382 | Adult feces |
| | | KBL384 | |
| | | KBL385 | |
| | *Lactobacillus paracasei* subsp. *tolerans* | KBL383 | Adult feces |
| | *Lactobacillus salivarius* | KBL389 | Adult feces |
| | | KBL391 | |
| | | KBL395 | |
| | | KBL397 | |
| | *Lactobacillus plantarum* subsp. *plantarum* | KBL396 | Adult feces |
| | *Lactobacillus acidophilus* | KBL402 | Adult feces |
| | | KBL409 | |

**3. Bacteria counting**

The bacteria samples were pelleted by centrifugation (Eppendorf, Hamburg, Germany) at 16000 g for 5 minutes and washed with 1× phosphate-buffered saline (PBS; pH 7.4, Biosesang, Seongnam-si, South Korea). After preparing the bacteria suspensions with 1 ml 1× PBS, 5 µl bacteria suspension, 0.75 µl SYTO 9 staining reagent, 0.75 µl propidium iodide, 5 µl microsphere standard and 488.5 µl 1× PBS were added to flow cytometry tubes according to the Bacteria Counting Kit (Molecular Probes, Eugene, OR, USA), and analyzed by flow cytometry (BD Accuri$^{TM}$ C6 Flow Cytometer, BD Biosciences, San Jose, CA, USA). Data was processed by framing regions around the various populations in the cytogram and the numbers of events in the bead region were counted to accurately estimate the volume analyzed in the data file. Calculating formula:

$$bacteria/ml = \frac{(\text{number of events in live bacteria region})\times(\text{dilution factors})}{(\text{number of events in bead region})\times 10\text{^}(-6)}$$

**4. The measurement of transepithelial electrical resistance**

Caco-2 cells were seeded onto Transwell® inserts (pore size 0.4 μm, Corning, NY, USA) at $3\times10^5$ cells/ml density according to the manufacturer's instructions, cultured for 7 days and changed with fresh medium every other day. Before bacterial treatment, Caco-2 cells were starved with FBS-free and antibiotic-free medium to synchronize the cell cycle. After 7 days of culturing, Caco-2 cells were treated with bacteria at multiplicity of infection (MOI) of 100 when the cells were differentiated. The integrity of the Caco-2 cell monolayers was evaluated at 0 h, 12 h, and 24 h by measuring the transepithelial/transendothelial electrical resistance (TEER) using an EVOM resistance meter (World Precision Instruments, Sarasota, FL, USA). Electrical resistance was measured until the temperature of plates was similar to room temperature and duplicate measurements were recorded for each sample. TEER change was calculated using the following formula:

$$TEER\ change = \frac{\text{TEER (after treatment)} - \text{TEER (before treatment)}}{\text{TEER (before treatment)}}$$

$$Relative\ TEER\ change = \frac{\text{TEER change (probiotic treatment)}}{\text{TEER change (control)}}$$

1

**5. Cell viability**

This viability assay is based on the ability of viable cells to incorporate and bind neutral red [22]. Caco-2 cells grown on Transwell® (Corning, NY, USA) were washed twice with PBS after incubation with probiotics for 12 h. Neutral red (Sigma-Aldrich, St Louis, MO, USA) was diluted in cell culture medium at the final concentration of 33 μg/ml and added to the wells for 2 h at 37°C. Then neutral red was extracted from the cells with 1% acetic acid (DUKSAN, Ansan-si, South Korea) - 50% ethanol (Merck Millipore, Billerica, MA, USA) on a plate shaker for 10 min. Extracts were transferred to a 96-well plate (SPL, Pocheon-si, South Korea). The neutral red content was measured using a spectrofluorometer (Infinite M200, TECAN, Männedorf, Switzerland) with excitation and emission wavelengths of 530 and 645 nm, respectively. The readings were expressed as percentages of the control cells.

**6. Western blot**

After 7 days' culture, Caco-2 cells were treated with tested probiotic strains at MOI of 100 or FBS-free and antibiotics-free Minimum Essential Media (Thermo Fisher Scientific, Waltham, MA, USA) only for 12 h. At the end of the experimental period, cells were washed twice with cold 1× PBS and lysed by 1× RIPA buffer (Rockland Immunochemicals, Limerick, PA, USA). Monolayers were scraped and the cell lysates were placed in pre-cooled microtubes. After centrifuging at 16000 g (Eppendorf, Hamburg, Germany) for 20 min at 4°C, the total proteins were quantified using Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts (5 μg) of total proteins were resolved on 8% SDS-polyacrylamide gels and the polyvinylidene fluoride membranes (GE Health, Chicago, IL, USA) were then incubated overnight at 4°C with rabbit anti-ZO-1, mouse anti-Occludin (ThermoFisher, Waltham, MA, USA) or mouse anti-GAPDH (Young In Frontier, Seoul, Korea) diluted in 5% skim milk. After three washes with 1× tris-buffered saline and polysorbate 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (ThermoFisher, Waltham, MA, USA). Bands were visualized using enhanced chemiluminescence reagent (Bio-Rad

Laboratories, Hercules, CA, USA) in G:BOX (Syngene, Bengaluru, India) and band intensity was quantified by Gene Tools analysis software (Syngene, Bengaluru, India) and band intensities were normalized by GAPDH.

**7. Confocal laser scanning microscopy**

After 7 days' culture, Caco-2 cells were treated with tested strains at MOI=100 or FBS-free and antibiotics-free Minimum Essential Media (Thermo Fisher Scientific, Waltham, MA, USA) only for 12 h. At the end of the experimental period, cells were washed twice with cold 1× PBS. Caco-2 cell monolayers were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% triton X-100 (AMRESCO, Solon, OH, USA) for 5 min on ice. Then cell monolayers were incubated overnight at 4°C with rabbit anti-ZO-1 and mouse anti-Occludin (ThermoFisher, Waltham, MA, USA). The cells were then incubated for 1 h with corresponding FITC-conjugated secondary antibodies, Alexa fluor 594 goat anti-mouse (Red; Molecular Probes, Eugene, OR, USA) and Alexa fluor 488 goat anti-rabbit (Green; Molecular Probes, Eugene, OR, USA) at room temperature in the absence of light. Stained cells were imaged by confocal laser scanning microscopy (Confocal-FV1000, OLYMPUS, Tokyo, Japan).

**8. Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Data were analyzed with Prism using t-test followed by Mann-Whitney test to compare tested bacteria strain treatment group with control group. Statistical significance was given as *$p < 0.05$, **$p<0.01$.

## III. Results

### 1. Screening of probiotic candidates that tighten the intestinal barrier.

Twenty-one strains of probiotic candidates were screened by the TEER assay to evaluate their effects on intestinal barrier (Table 2). Figure 1 shows relative change in TEER of 9 different species of 21 strains in *Lactobacillus* spp. and the numbers of scatter dots represent the numbers of strains in the same species. There was just one stain of *L. reuteri*, *L. paracasei* and *L. plantarum* that were difficult to investigate the effects of *Lactobacillus* species on TEER change. However, *L. rhamnosus* of 5 strains showed significant beneficial effects on TEER change. Amongst the 21 strains, KBL363 (*L. rhamnosus*), KBL365 (*L. rhamnosus*) and KBL385 (*L. casei*) induced the most increase in TEER, while no significant differences among candidates were observed. K342 and KBL346 seemed to have decreased TEER change compared to the non-treatment control group, however, these changes were not significant. *E. coli,* used as a negative control, induced a significant decrease in TEER change about 8.22 ± 4.10 times relative to the non-treatment control, indicating intestinal barrier disruption (Table 2).

**Table 2. TEER change of 21 strains of *Lactobacillus* spp.**

| Genus | Species | Strain | Relative TEER change[a] |
|---|---|---|---|
| *Escherichia* | *Escherichia coli* | *E. coli* O157 EC4115 | -8.22 ± 4.10 |
| *Lactobacillus* | *Lactobacillus gasseri* | KBL342 | 0.32 ± 0.17 |
| | | KBL381 | 1.20 ± 0.76 |
| | *Lactobacillus reuteri* | KBL346 | -0.39 ± 0.49 |
| | *Lactobacillus rhamnosus* | KBL351 | 2.20 ± 0.08 |
| | | KBL354 | 2.20 ± 0.72 |
| | | KBL362 | 3.70 ± 1.40 |
| | | KBL363 | 4.50 ± 0.66 |
| | | KBL365 | 4.30 ± 1.20 |
| | *Lactobacillus fermentum* | KBL374 | 3.00 ± 0.71 |
| | | KBL375 | 2.90 ± 1.70 |
| | *Lactobacillus casei* | KBL382 | 2.60 ± 0.66 |
| | | KBL384 | 2.60 ± 0.90 |
| | | KBL385 | 3.80 ± 0.49 |
| | *Lactobacillus paracasei* subsp. *tolerans* | KBL383 | 2.50 ± 0.29 |
| | *Lactobacillus salivarius* | KBL389 | 1.60 ± 0.64 |
| | | KBL391 | 1.10 ± 1.60 |
| | | KBL395 | 1.80 ± 0.86 |
| | | KBL397 | 1.30 ± 0.35 |
| | *Lactobacillus plantarum* subsp. *plantarum* | KBL396 | 3.30 ± 0.76 |
| | *Lactobacillus acidophilus* | KBL402 | 2.80 ± 0.82 |
| | | KBL409 | 2.50 ± 0.06 |

[a]Control:1 ± 0.79; treated with bacteria for 24h.

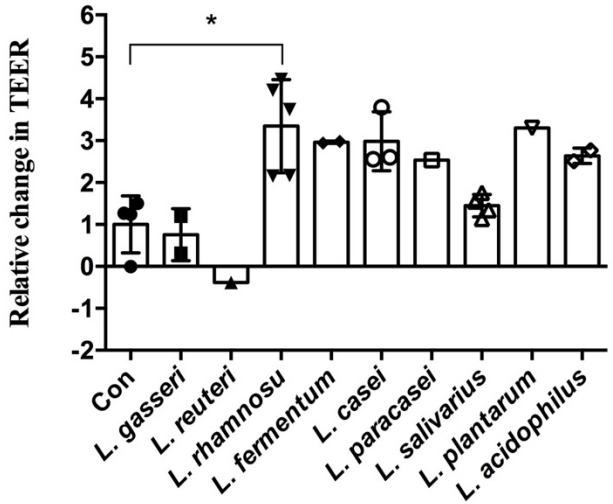



**Figure 1. TEER change of 9 different *Lactobacillus* species.**

Caco-2 cells were treated with tested bacteria at MOI=100 for 24 h. Experiments were carried out in duplicate and data represent the mean of relative change in TEER ± SD. Statistical differences were calculated by t-test. (*$p < 0.05$)

**2. Repeated beneficial effects of probiotic candidates on the intestinal barrier.**

According to screening data, we selected 3 probiotic candidates which had the most increased TEER change and the 3 strains were KBL363 (*L. rhamnosus*), KBL365 (*L. rhamnosus*) and KBL385 (*L. casei*). To confirm the sustainable effects on the intestinal barrier, 3 repeated TEER assays were conducted with 3 probiotic candidates at 12 h and 24 h post-treatment. As shown in Figure 2, KBL365 and KBL385 significantly increased TEER change 12 h after probiotic treatment, while KBL363 tended to enhance TEER change as well. The beneficial effects of probiotic candidates were sustained for 24 h and KBL385 showed significant changes in TEER regulation. However, the tightening effects were more obvious at the 12 h time point, thus 12 h was the fixed probiotic treatment time point in following experiments (Figure 2).

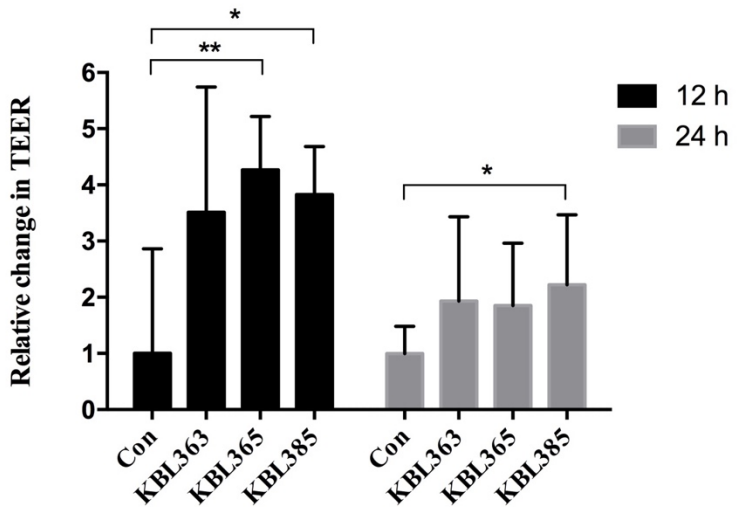



**Figure 2. Repeated TEER assays with probiotic candidates.**

Caco-2 cells were treated with probiotic candidates at MOI=100 and TEER was measured at 12h and 24 h. Data represent the means of relative change in TEER ± SD in 3 independent experiments carried out in duplicate or triplicate. Statistical differences were calculated by t-test. (*$p < 0.05$, **$p < 0.01$)

**3. Effects of probiotic candidates on Caco-2 cell viability.**

If administration of probiotic candidates caused damage to Caco-2 cells, or increased live Caco-2 cell numbers, the experimental data would be unreliable to demonstrate the benefits of probiotics. To investigate whether any of the probiotics would affect the Caco-2 cell viability, a cell viability test was performed after 12 h treatment of probiotics. As shown in Figure 3, no probiotic candidates significantly affected the cell viability following 12 h of incubation when MOI=100 was tested.

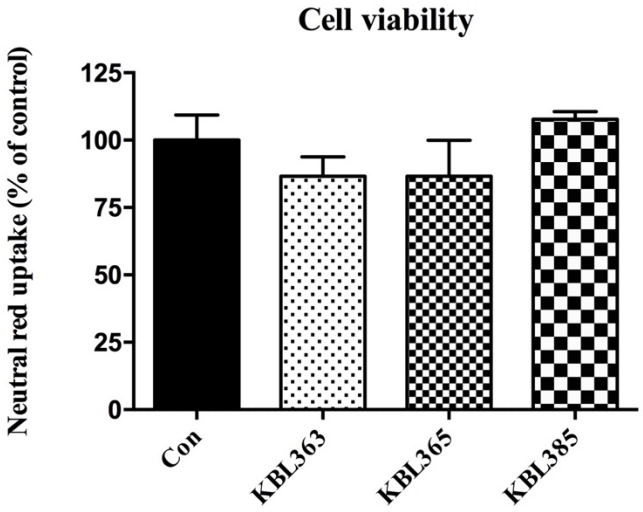
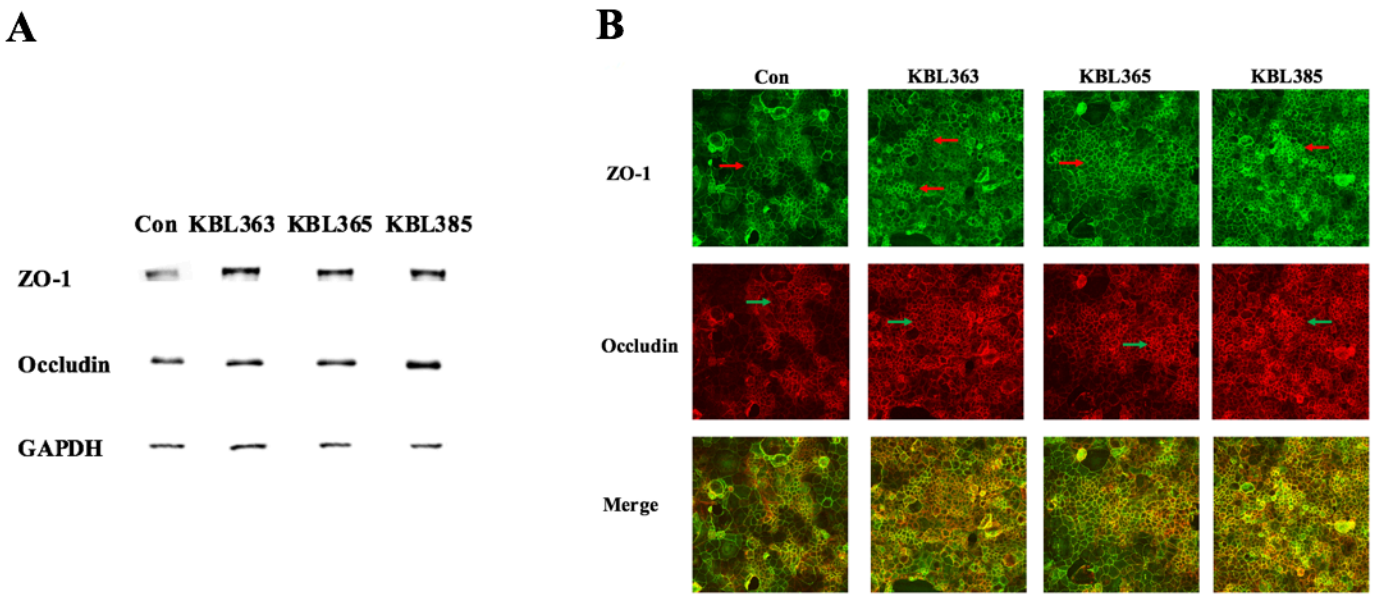



**Figure 3. Relative cell viability after probiotics treatment to Caco-2 cells.** The viability of Caco-2 cells was assessed by neutral red uptake assay followed by 12 h treatment of live bacteria at MOI=100. This experiment was carried out in triplicate and data represent the means of relative change in mean ± SD. These was no statistical significance observed between control and selected bacteria strain by t-test.

**4. Effects of probiotic candidates on tight junction proteins ZO-1 and Occludin.**

The results of ZO-1 and Occludin protein expression fold changes showed that in agreement with a significant increasing in TEER change, there were obvious increases in ZO-1 and Occludin protein level by probiotic candidate administration (Figure 4). 3 strains of *Lactobacillus* spp. (KBL363, KBL365, KBL385) significantly increased ZO-1 protein expressions about 1.5 to 2 times that of the control group, while Occludin protein expressions was up-regulated approximately 1.5 times relative to the control group. However, there were no differences among tested *Lactobacillus* spp. as well as TEER assay (Figure 4C, D).

To visualize protein regulation, an immunofluorescence assay was conducted with different probiotic candidates. As Figure 4B shown, when ZO-1 and Occludin merged, immuno-fluorescence turned yellow, indicating that ZO-1 and Occludin were both localized on the boundary of the cell membrane and tightening the transmembrane space. In accordance with the western blot data, probiotic treated Caco-2 cells displayed higher intensity of antibodies than the control group.


A
Con
KBL363
KBL365
KBL385
ZO-1
Occludin
GAPDH
B
Con
KBL363
KBL365
KBL385
ZO-1
Occludin
Merge

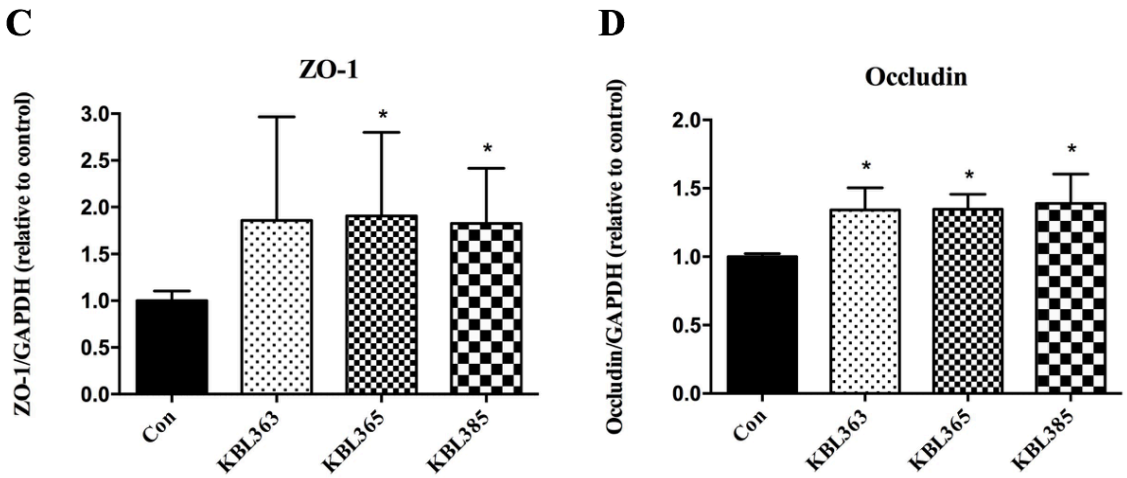
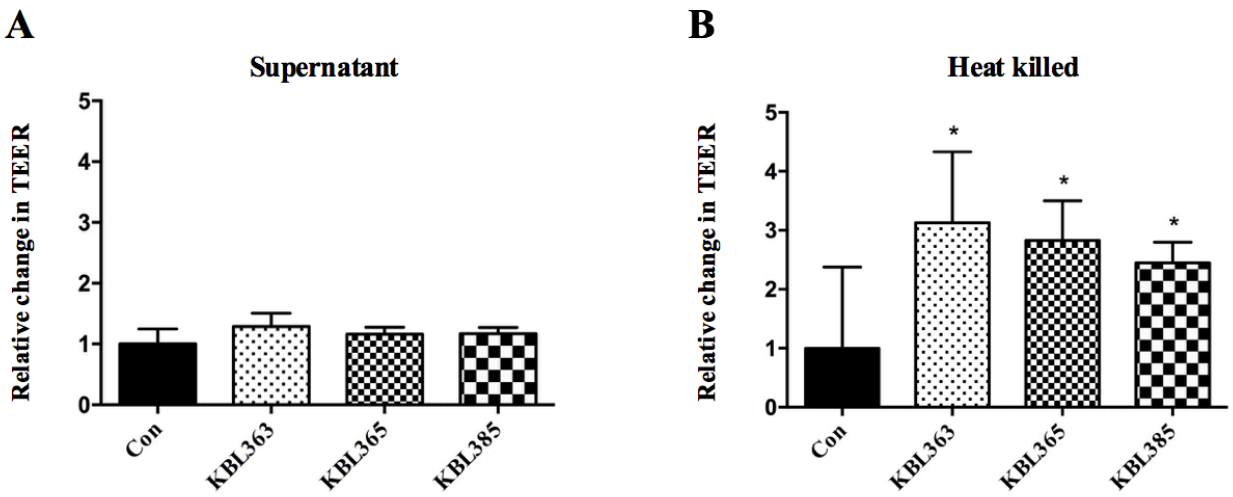



**Figure 4. Effects of *Lactobacillus* spp. on tight junction proteins.**

(A)Band intensities of ZO-1 and Occludin proteins assessed by western blot (B) Visualization of tight junction proteins after probiotic treatment. Samples were stained for Occludin in red and ZO-1 in green. Confocal images were acquired with an Olympus laser scanning microscope using 20× lens. (C) Relative fold change of ZO-1 normalized by GAPDH in 3 independent experiments. (D) Relative fold change of Occludin in 3 independent experiments.

**5. Effects of culture supernatant and heat-killed *Lactobacillus* spp. on intestinal barrier.**

To investigate whether dead probiotics or probiotic culture supernatant increase intestinal barrier, a TEER assay was conducted in 2 repeated independent experiments. Multiple publications demonstrated that bacterial supernatant as well as live probiotics had beneficial effects due to the short chain fatty acid content in culture supernatant [20]. However, supernatant of KBL363, 365, 385 did not increase TEER change (Figure 5A). Instead, heat-killed *Lactobacillus* spp. retained the tightening effects significantly in TEER change (Figure 5B). In accordance with TEER data, heat-killed KBL363, KBL365 and KBL385 increased tight junction proteins ZO-1 and Occludin significantly compared to the control group (Figure 5C, D, E).


A
Supernatant
Relative change in TEER
0
1
2
3
4
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Heat killed
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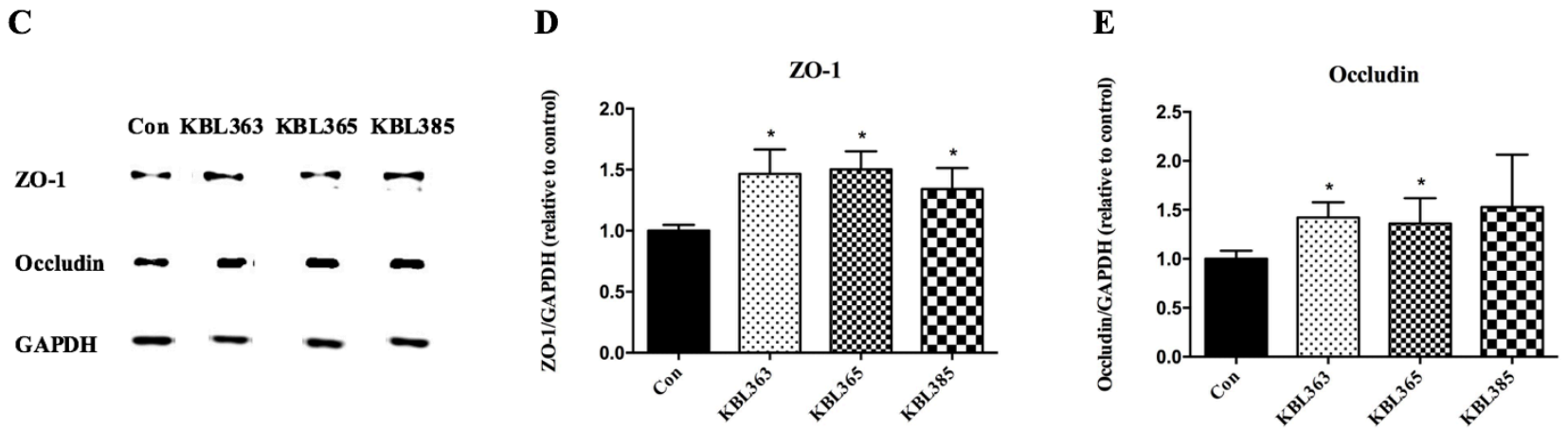


**Figure 5. Effects of culture supernatant and heat-killed *Lactobacillus* spp. on the intestinal barrier.**

(A, B) Caco-2 cells were treated with probiotic supernatant or heat-killed probiotics for 12 h at MOI = 100. Data represent the relative change in average ± SD of two independent experiments. (C). ZO-1 and Occludin proteins were calculated by western blot assay (D, E). Band intensities were assessed by Syngene. Data represent the relative fold change normalized by GAPDH. Statistical differences were calculated by one sample t-test, *$p < 0.05$ (C).

## IV. Discussion

The data presented in this study showed that different strains of *Lactobacillus* spp. had various effects on TEER change and we had screened out probiotic candidates that tightened the intestinal barrier. After repeated independent experiments to confirm sustainable beneficial effects of *Lactobacillus* spp., probiotic candidates KBL363 (*L. rhamnosus*), KBL365 (*L. rhamnosus*) and KBL385 (*L. casei*) significantly increased TEER change about 4 times that of control group. There are probiotic products available now whose species also belong to *L. rhamnosus* and *L. casei* [23, 24]. *L rhamnosus* GG is one of the most researched probiotics that increased TEER change about 4 times that of control [25]. Probiotic enhancement of intestinal barrier is affected by protein expression in the tight junction signaling pathway, particularly ZO-1 and Occludin [26]. We found that probiotic candidates increased tight junction proteins ZO-1 and Occludin in accordance to up regulation in TEER change. Decreased intestinal barrier has become a key causal effect in gut dysfunction and related inflammatory gut diseases [27]. Probiotics have become the subject of a great deal of investigation. Probiotic therapy is based on the concept of normal healthy microflora

and abundant evidence implies that specific strains selected from healthy gut microflora exhibit powerful capabilities [28]. *In vitro* TEER assay has been a typical method to characterize beneficial effects of probiotics on the intestinal barrier [29], however, operators should be cautious about experimental practice because TEER assay results are changed rapidly in response to temperature [30].

Recently there are an increasing number of studies about the mechanisms through which probiotics boost beneficial effects which indicate that the immune system plays an important role in mediating host-microbiota interactions [31]. However, it was hard to investigate immune regulations in Caco-2 cell line, for defects *in vitro* studies. Probiotics are recognized by TLRs and activate NF-κB to induce dendritic cells to produce different anti-inflammatory and pro-inflammatory cytokines mediating immune reactions [32]. However, unlike the immune system and intracellular signaling, there are few findings about which components of probiotics activate the host signaling. Cell-free supernatant of *Lactobacillus casei* and *Lactobacillus rhamnosus* GG increased level of tight junction protein ZO-1 [33]. To investigate the effects of the culture supernatant of the *Lactobacillus* spp. in our study, we tested the functions of supernatant in TEER assay with

the tested probiotic candidates. However, in this study supernatant of *Lactobacillus* spp. seemed to have no significant tightening effects on the intestinal barrier. Yan et al. (2007) illustrated that soluble proteins p40 and p75 in probiotic culture supernatant might be a key factor in beneficial effects of *Lactobacillus* spp. [33]. The way bacteria interact with host is various and the component might be short chain fatty acid or other proteins.

Recently, heat-killed *Akkermansia muciniphila* and a purified membrane protein were shown to improve metabolism in obese mice [35]. This finding shows that specific molecules isolated from probiotic membranes can have similar effects on health associated phenotypes as the probiotic itself. Thus, we confirmed that heat-killed *Lactobacillus* spp. in our study retained tightening effects on intestinal barrier. KBL363, KBL365 and KBL385 were shown to increase TEER change as well as tight junction proteins significantly. According to the World Health Organization (WHO), probiotics are recognized as live bacteria [36]. Research has indicated that compared to live probiotics, heat-killed bacteria had reduced effects on cytokine regulation [37]. In this study, in comparison to the effects of live bacteria on TEER change, dead bacteria had reduced effects in TEER change and ZO-1 expression, indicating

tightening effects on intestinal barrier is the result of multiple components' signaling in tight junctions.

This study screened probiotic candidates, investigated their effect on intestinal barrier functionality and highlighted that tightening effects were retained in heat-killed bacteria. Further targeted studies will investigate more specific components of probiotic and investigate their effects *in vivo* and moreover in clinical trials. The long-term aim is to understand the mechanisms in molecular signaling in improving effects on human health and investigate a beneficial probiotic as a new disease treatment.

## V. References


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## 국문초록

# 장보호막을 강화시키는
# 락토바실러스 균주의 선별과 평가

서울대학교 보건대학원
환경보건학과 환경보건학전공
MEINA LIN

지도교수 고 광 표

장보호막은 영양물질의 흡수, 면역 조절, 유해물질에 대한 방어막이 되어 주기 때문에 인간과 장내 미생물의 상호 작용에서 중요한 역할을 한다. 염증성 장 질환의 치료 방법으로 장내 미생물 중 안전성이 검증된 락토바실러스에 대한 연구가 많이 진행되고 있다. 하지만 균의 유익한 효과에 대한 반복적인 증거와 재현성에 대한 연구는 더 진행 되어야 한다. 본 연구는 1) 한국인 분변 유래 21종의 락토바실러스를 이용하여 반복적인 transepithelial/transendothelial electrical resistance (TEER) assay를 통하여 장보호막을 강화시키는 프로바이오틱스 후보 균주를 선별하고 2) 선별된 균주가 장보호막 조성 단백질 ZO-1 과 Occludin에 미치는 영향을 평가하고 3) 균주 배양 상층액과 열처리를 통한 사균을 이용하여 균주의 어떠한 물질이 장보호막에 유익한 영향을 미치는지에 관하여 연구를 진행하였다. 반복적인 TEER assay를 통하여 장보호막 강화 효과를 보여주는 *L. rhamnosus* (KBL363, KBL365) 와 *L. casei* (KBL385)를 선별하였다. 선별된 균주는 TEER 결과와 일치하게 ZO-1과 Occludin 단백질을 유의미하게 증가하였다. 본 연구의 균주 배양액은 장보호막에 유익한 효과가 없었지만

열처리를 통한 사균은 장보호막 강화 효과가 있었다. 열처리를 통한 균은 활성은 없지만 세포 외부의 단백질 등의 활성이 유지되어 있기에 세포 외부의 물질에 의한 장보호막 강화 효과가 있었다는 것을 확인 할 수 있었다. 결론적으로 본연구는 장보호막 강화 효과가 있는 3개의 균주를 확보하였고 사균도 생균과 비슷한 장보호막 강화 효과가 유지된 것을 통하여 세포 외부 물질에 의한 강화효과임을 알 수 있다.

**주요 단어:** 장보호막, 릭토바실러스, 균배양 상층액, 사균

**학번:** 2015-24069