Probiotics have been reported to be effective in maintenance of intestinal homeostasis and prevention or treatment of infectious diseases, including rotaviral infections. Rotavirus changes the function of the small intestine epithelium and thereby causes lesions mainly in digestive organs, resulting in vomiting, fever and mild diarrhea. The aim of the present study was to clarify the probiotics’ mechanisms of immune response enhancement and viral activity inhibition. Lactobacillus ruminis Lb0211 and Bifidobacterium longum Bf1206 were selected by screening antiviral activity and determining the antiviral mechanisms of each strain. The selected strains induced the expression of chemokines, including interleukin-8, monocyte chemoattractant protein-1 and type 1 interferons (IFN-α and IFN-β) in Caco-2 cells. These chemokines and cytokines may promote the immune response and inhibit viral replication. Therefore, the strains selected in this study can be alternative treatments for rotaviral infection.

**Keywords:** Bifidobacterium, Lactobacillus, anti-rotaviral, probiotics

Rotaviral infections occur worldwide and cause severe diarrhea in infants and children (Chen et al., 2012; Vlasova et al., 2013). These infections occur most frequently among infants and children under 5 years old. Early treatment is important because rotaviral infections are characterized by a wide range of clinical symptoms. Rotavirus changes function of the small intestine epithelium and thereby causes lesions mainly in digestive organs; while some patients are asymptomatic, others may experience fever, mild diarrhea, and vomiting. In severe cases, excessive diarrhea may lead to electrolyte imbalance, life-threatening dehydration, and death (Sen et al., 2020). Vaccines are not always sufficient to prevent diseases caused by human rotaviruses (HRVs) because the high costs of vaccine programs make them difficult to be implemented in low-income countries.

Probiotics are often used as alternative treatments that relieve infectious diarrhea and maintain intestinal homeostasis (Preidis et al., 2011; Angel et al., 2012). Probiotics containing Lactobacillus, Bifidobacterium, and Streptococcus species are reported to stimulate the proliferation of epithelial cells and promote early immune responses (Patel and DuPont, 2015). Recent studies conducted on Bifidobacterium and Lactobacillus demonstrate that they may inhibit the adhesion and replication of rotavirus
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and thus prevent the spread of infection (Muñoz et al., 2011). Vlasova et al. (2013) reported that *Lactobacilli* and *Bifidobacterium* strains inhibit rotaviral infection by interfering with viral attachment. Fernandez-Duarte et al. (2018) also reported the anti-rotaviral effects of *Bifidobacterium adolescens* and *Lactobacillus casei*, which can be used as bacteriotherapeutic probiotics.

In this study, we selected *Lactobacillus ruminis* Lb0211 and *Bifidobacterium longum* Bf1206 as probiotics due to their expected anti-rotaviral effects. To determine the antiviral mechanisms of probiotics, we measured the changes in chemokines and antiviral cytokines, interferons, secreted during rotaviral infection. Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) were selected as representative chemokines. We measured the changes in levels of these chemokines among cells treated with probiotic lactic acid bacteria (LAB), sonicated-Lb0211 and Bf1206 and compared them with cells infected with rotavirus alone.

**Materials and Methods**

**Materials**

General Anaerobic Medium (GAM) (Nissui Pharm. Co. Ltd.), Roswell Park Memorial Institute (RPMI)-1640, minimum essential medium (MEM) (GIBCO), fetal bovine serum (FBS) (GIBCO), and penicillin (10,000 U/ml) and streptomycin (10,000 U/ml) (GIBCO) were used. We obtained 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich, Inc. (Sigma-Aldrich). Human IL-8 enzyme linked immunosorbent assay (ELISA) and Human MCP-1 ELISA sets were purchased from BD Biosciences (BD).

**Probiotics strains and preparation of sonicated–LAB**

Probiotics were obtained from SahmYook Probiotics Research Institute. These strains were cultivated in GAM under anaerobic conditions (90% N2, 5% H2, 5% CO2, 37°C) for 48 h in the Bactron Anaerobic Chamber (Sheldon Manufacturing Inc.). The strains were centrifuged and washed with PBS. The washed probiotics were resuspended in PBS at a concentration of 1 × 10⁹ colony forming units (CFU)/ml, and the cells were sonicated under the following conditions: 100% amplitude, pulse on-60 sec, and pulse off-60 sec, with a total of six cycles (Sonicator, VCX 130, Sonic & materials Inc.). The sonicated cells were filtered through a 0.22 μm filter and the filtrate was used in the experiment.

**Rotavirus and cell lines**

Caco-2 and HT-29 cell lines were purchased from the Korean Cell Line Bank (KCLB®). HT-29 cell lines were mixed with 1% penicillin (10,000 U/ml) and streptomycin (10,000 U/ml) in RPMI-1640 medium containing 10% FBS and cultured at 37°C under 5% CO2 in a constant temperature incubator (MCO-18AIC, SANYO). Caco-2 cell lines were mixed with the same concentrations of penicillin and streptomycin in MEM containing 10% FBS and cultured in the incubator. The HRV “Wa” strain (HRV Wa strain, KUMC-47) was purchased from the Korea Bank for Pathogenic Viruses.

**Cytotoxicity test**

The MTT assay was performed to verify the cytotoxic activity of sonicated-LAB. Each cell line was seeded in a 96 well plate (1 × 10⁴ cells/well) and incubated for 24 h. Then, cells were treated with the probiotic Lb0211, Bf1206 and cultured for 24 h. The supernatant was removed, and cells were incubated in 100 μl of MTT solution for 4 h at 37°C. The formazan was dissolved in 100 μl of dimethyl sulfoxide, and the absorbance was measured at 540 nm using an ELISA microplate reader (Molecular devices).

**Plaque assay**

Caco-2 cells were seeded at a density of 1 × 10⁴ cells/ml and cultivated until >90% of cells attached to the bottom of the well. The rotavirus solution was diluted via ten-fold serial dilutions and incubated for 50 min at 37°C with the infection media (trypsin 5 μl/ml, Dulbecco’s modified eagle medium, DMEM). During the activation of the virus, the media were removed and washed with PBS to prevent trypsin neutralization by the serum. The activated rotavirus was spread evenly at the surface of the cells (400 μl/well) and incubated at 37°C under 5% CO2 for 2 h. The cells were shaken every 10 to 15 min so that they could respond evenly to the virus without drying. After 2 h, the viral fluid was removed, and the cells were
washed twice with PBS. Next, 2 ml of agarose overlay medium (3% [w/v] agarose: 2.5% FBS-RPMI = 1:2) was added per well and allowed to harden at room temperature. Then, the cells were incubated at 37°C under 5% CO₂ until plaques were formed. When plaque formation was observed, 1 ml of 10% formaldehyde was added to each well and fixed for 1 h. Then, the fixation and agarose overlay medium was carefully removed to prevent the fixed cells at the bottom from falling off. The viral titer was calculated via the following equation by counting clear zones after staining with neutral red or crystal violet solution.

\[ \text{Viral titer (plaque-forming units/ml)} = (\text{number of plaques per well}) \times (\text{reciprocal of dilution rate}) \times (\text{reciprocal of infected viral fluid}) \]

Screening and evaluation of antiviral effects of probiotic Lb0211 and Bf1206

The plaque assay was performed to screen the anti-rotaviral effects of probiotic Lb0211, Bf1206 and select the optimal strain. Caco-2 cells were seeded using the same method as the plaque assay. At the same time, cells were treated with the probiotic Lb0211 and Bf1206 (1 × 10⁹ CFU/ml; 40 μl/well), incubated for 2 h, and then infected with rotavirus. The positive control group was treated only with the medium, while the negative control group was treated only with rotavirus. The antiviral effects of the selected strains were evaluated using the same method.

Measuring the release of IL-8 and MCP-1

After infecting the host cells with rotavirus, we collected the culture supernatant and ELISA was used to quantify the release of IL-8 and MCP-1. The culture supernatant (100 μl) was added to a microplate coated with the primary antibody on the previous day, incubated at room temperature for 2 h, and washed five times with a wash buffer. After washing, the plates were treated with 100 μl of secondary antibody solution for 1 h. After incubation with the secondary antibodies, the substrate solution was added, and the plate was incubated in the dark for 30 min. The stop solution was added to quench the reaction, and absorbance was measured at 450 nm. The calibration curves were created using serially diluted solutions containing human IL-8 and MCP-1 and used to quantify chemokine release in the supernatants.

### Table 1. Primer sequences for RT-q PCR

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Polarity</th>
<th>Nucleotide sequences</th>
</tr>
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<tbody>
<tr>
<td>IL-8</td>
<td>Sense</td>
<td>GGCTCTCTTGGCAGCTCTCTCTTGGCAGCTCCTCCAAACACCTCCTGCAACCA</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>CTCTCTCTTGGCAGCTCTCTCTTGGCAGCTCCTCCAAACACCTCCTGCAACCA</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Sense</td>
<td>CTGAAGGCTGAGCCTCTCCTGACTCTGCAACAG</td>
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<td></td>
<td>Antisense</td>
<td>CAAAACATCACACCTGAGGTTGAGAAGTCAACCA</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Sense</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GCACATATGACACACCTGATTCAGAGAG</td>
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<tr>
<td>IFN-β</td>
<td>Sense</td>
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</tr>
<tr>
<td>GAPDH</td>
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<td></td>
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<td>CCTGCTCACCACCTTCTTG</td>
</tr>
</tbody>
</table>

Real–time quantitative PCR (RT–q PCR)

QIAamp® RNA Viral Mini Kit (Qiagen), Omniscript® Reverse Transcription kit (Qiagen), Power Master SYBR® Green PCR Master Mix (Applied Biosystems), and Applied biosystems® Step One Plus™ Real-Time PCR Systems (Applied Biosystems) were used for RT–q PCR.

After the Caco-2 cells were infected for 24 h, the cells and the supernatant were collected by trypsinization and centrifuged for 5 min at 1,500 rpm. Total RNA was extracted using RNeasy mini kit and used as a template for cDNA synthesis. The primers for CXCL8 (IL-8), MCP-1, and the Type 1 Interferons (IFN-α and IFN-β) are listed in Table 1. The relative gene expression was calculated using the 2⁻ΔΔCT method. GAPDH, a housekeeping gene, was used as an internal control (Livak and Schmittgen, 2001).

\[ \text{Amount of target} = 2^{-\Delta\Delta CT} \]

\[ \Delta\Delta CT = (C_{T,\text{Target}} - C_{T,\text{GAPDH}})_{\text{Sample}} - (C_{T,\text{Target}} - C_{T,\text{GAPDH}})_{\text{Control}} \]

Statistical analysis

All the experimental results are expressed as the standard errors of means (SEM). IBM SPSS version 22 was used for all statistical analyses. Differences were considered statistically significant at a \( p \)-value < 0.05.

Results

Cytotoxicity test

*Bifidobacterium adolescentis* Bf1205 and Bf1206 yielded the lowest and highest survival rates of 76.69 ± 6.72% and
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98.79 ± 1.95%, respectively, in Caco-2 cells. The cell viability in Caco-2 cells, survival rates were shown to be more than 80%, except for *Bifidobacterium longum* Bf1205 (Fig. 1). The individual sonicated-LAB was serially diluted for treatment of the Caco-2 cells. Viability was especially related to toxicity of probiotic Lb0211 and Bf1205 by MTT assay, and no difference in concentration and cell toxicity was found.

**Antiviral effects of probiotic Lb0211 and Bf1206**

*Lactobacillus ruminis* Lb0211 and *Bifidobacterium longum* Bf1206 were selected through screening. The antiviral effects of the two strains were evaluated at concentration of $1 \times 10^9$ CFU/ml (Fig. 2). Both strains showed higher antiviral efficacy in Caco-2 cells as compared to the HT-29 cells (data not shown), and this effect was concentration-dependent. Therefore, we chose to use Caco-2 cells for the rest of the experiments. When Caco-2 cells were treated with $1 \times 10^9$ CFU/ml of Lb0211, the inhibition rate was about 55%.

**Changes in chemokines by probiotics Lb0211 and Bf1206**

Although the change in IL-8 and MCP-1 release only slightly increased shortly after the infection, there was a time-dependent increase in IL-8 and MCP-1 secretions (Figs. 3B and 4B).
These results suggest that the secretion of IL-8 and MCP-1 increases as the rotavirus replicates in the cells. Experiments at the molecular level also confirmed that mRNA expression of IL-8 and MCP-1 increased compared to the control group (Figs. 3A and 4A). After treatment with Lb0211, the expression of IL-8 increased approximately 2.4-fold and that of MCP-1 increased 1.3-fold. IL-8 expression increased 1.9-fold and MCP-1 expression increased 2-fold after treatment with Bf1206.

**Antiviral action mechanism of probiotic Lb0211 and Bf1206**

Rotaviral infection is reported to increase the expression of type 1 IFNs, which regulate the response to viral infections in humans (Holloway and Coulson, 2013). In accordance with this, we observed changes in IFN-α and IFN-β after treatment with the selected strains. The mRNA expression of IFN-α increased 1.5-fold in treated with Lb0211, and IFN-β mRNA expression was increased in both groups compared to the control (Fig. 5).

**Discussion**

Probiotic bacteriotherapy, which involves using beneficial species to replace pathogens, is suggested as an alternative treatment for infectious diseases (Preidis et al., 2011; Chen et al., 2012; Vlasova et al., 2013). Among probiotics, studies are currently being conducted using Lactobacillus and Bifidobacterium species. These studies focus on the ability of these probiotics to inhibit the adhesion and replication of rotavirus or prevent
infection in vitro. Ventola et al. (2012) reported that Lactobacillus rhamnosus GG increased the removal of rotavirus in vivo. Also, it was reported that the percentage of rotavirus-infected MA 104 cells decreased when treated with L. casei, L. fermentum, B. adolescentis, and B. bifidum and their metabolite products (Fernandez-Duarte et al., 2018). However, the anti-rotaviral mechanisms of probiotics are not currently well understood.

In previous study, we conducted a plaque assay using Vero cells to screen the antiviral effects of isolated probiotic strains (data not shown). We selected Lactobacillus ruminis Lb0211, and Bifidobacterium longum BF1206, and evaluated their anti-rotaviral effects. Although the effects of these probiotics were slightly different across cell lines, the antiviral effects were concentration-dependent in both the cell lines.

During acute and chronic inflammation, IL-8 attracts neutrophils and MCP-1 attracts T lymphocytes and monocytes to the areas of infection (Yoshimura, 2017; Meniallo et al., 2018). These results showed that IL-8 and MCP-1 increased with the replication of the rotavirus, thus indicating that the activity of immune cells increases as the infection progresses. Furthermore, the expression of IFN-α increased after treatment with Lb0211, and IFN-β increased in both treatment groups as compared to the control.

Changes on the production of chemokines after rotaviral infection showed results to the Caco-2 cell experiments. The expression of IL-8 and MCP-1 increased in both probiotics-treated as compared to the control, thus indicating an immune response.

The expression of type 1 IFN was significantly increased in the treated as compared to the control, which was only infected with rotavirus. Rotavirus is known to inhibit the expression of IFNs (Shen et al., 2013). Previous reports showed that IFN-deficient mice were more sensitive to rotaviral infectious and showed a higher rate of viral replication than that of the wild-type (Angel et al., 2012). Furthermore, viral replication was reduced following IFN-γ treatment (Hernández et al., 2015). As such, IFNs are important factors that affect viral replication. Probiotics may inhibit rotaviral replication by inducing the expression of type 1 IFNs.

Lb0211 and BF1206 may be effective alternative treatments for rotaviral infections that cause minimal side effects. Moreover, it is believed that they will be able to reduce the frequency of life-threatening complications, such as diarrhea and severe dehydration, by treating the infection.

Acknowledgments

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References


