Antiviral Properties of Probiotic Mixtures against Rotavirus in the Rat

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Rotavirus is a major cause of acute gastroenteritis in young children in developed and developing countries. The use of probiotics for the treatment of gastrointestinal diseases is both safe and easily accessible. In this study, we evaluated the anti-rotaviral activities of probiotic mixtures in a Sprague-Dawley rat. 24 litters with their dams were randomly assigned to four groups; placebo, phosphate buffered saline (PBS), and two probiotic mixture (PRO-1 and PRO-2) groups. All rats were inoculated with rotavirus at dose of 8 log plaque forming units per rat at 5 days old. Animals in the PRO-1 and PRO-2 groups were orally administered probiotic mixtures 1 or 2, respectively, at a dose of 8 log colony forming units daily during 4 days. For control purposes, placebo and PBS groups were orally administered the same amount of placebo (containing maltose and polydextrose) or PBS once daily for 4 days, respectively. Antiviral analysis was performed by real-time quantitative PCR (RT-qPCR) and observing intestinal villi. As a result, weights of small intestines were greater in the PRO-1, PRO-2 groups than in control groups. Villi were short and villous epithelial necrosis was exhibited in control groups, but these morphological changes were not observed in PRO-1, PRO-2 treated rats. RT-qPCR analysis showed that VP7 gene level of rotavirus in fecal samples and small intestinal epithelial cells were lower in the PRO-1 and PRO-2 groups. These findings suggest that probiotic mixtures may be useful probiotics for the treatment of or as alternative therapies for rotaviral gastroenteritis.

Keywords: anti-rotaviral activities, probiotics, real-time quantitative PCR (RT-qPCR)
the above-mentioned problems (Cha et al., 1999).

Probiotics are live microorganisms (Bae et al., 2002; Colbère-Garapin et al., 2007) that use carbohydrates and saccharides to produce organic acids. Probiotics are used in foods to promote host health (Dykstra et al., 2011). The use of probiotics for the treatment of gastrointestinal diseases has been shown to be safe and easily accessible (Rautava, 2007; Kotzampassi and Giamarellos-Bourboulis, 2012). And probiotics have been shown to prevent and to be useful for treating immune diseases (Majamaa et al., 1995; Rautava, 2007; Kotzampassi and Giamarellos-Bourboulis, 2012). Recently, study was reported (Majamaa et al., 1999). It was shown to prevent and to be useful for treating acute rotaviral diarrhea in infants and children (Shomikova et al., 1997; Chandra, 2002).

This study was performed to evaluate the effect of probiotic mixtures on rotavirus by animal tests used Sprague-Dawley infant rat. Body and small intestine weights were measured, small intestines were examined by light microscopy, and amounts of rotavirus in feces and small intestines were assayed by real-time quantitative PCR (RT-qPCR).

Materials and Methods

Cell lines and rotavirus

A group Rotavirus (Wa, ATCC VR-2018, Serotype1 G1) was grown in VeroE6 cell lines (CRL-1586, African green monkey). Trypsin (Type IX, Gibco) was used at a final concentration of 5 μl/ml for 50 min at 37°C to activate virus infectivity. After infection cells, they were placed in fresh medium containing 0.5 μl of trypsin per ml without FBS and incubated for 48 h in a 5% CO2 atmosphere at 37°C.

Probiotic mixture

Probiotic strains and the placebo were manufactured and supplied by Cell Biotech Co., Ltd, Korea. Probiotic mixture 1 contained six types of live probiotic bacteria, B. longum, B. lactis, L. acidophilus, L. rhamnosus, L. plantarum, and Pediococcus pentosaceus. Probiotic mixture 2 contained five types of live probiotic bacteria, B. longum, B. lactis, L. rhamnosus, L. plantarum, and Pediococcus pentosaceus. The placebo contained maltose and polydextrose. These probiotic mixtures and placebo were used for animal experiment after being suspended in phosphate buffered saline (PBS).

Animal experiment

24 Sprague-Dawley rats of either sex (2 days old) were purchased from Raonbio (Raonbio, Korea), and housed in a temperature-controlled animal room (22 ± 2°C/humidity 55 ± 5%) under a 12 h light/dark cycle. Food and water were provided ad libitum from the day of arrival until the completion of experiments. 24 litters with their dams were randomly assigned to four groups (6 rats per group); placebo, PBS, and probiotic mixtures (PRO-1, PRO-2) groups. The experiment was conducted after stable period of 3 days. All animals were inoculated with rotavirus at dose of 8 log plaque forming units per animal at 5 days old. From 1 day post inoculation (DPI 1) to DPI 4, animals in the PRO-1 and 2 groups were orally administered PRO-1 or 2 at 8 log colony forming units once daily for 4 days, respectively. For control purposes, the placebo and PBS controls were orally administered the same amount of placebo (containing maltose and polydextrose) or PBS once daily on the same 4 days.

Animals were monitored daily for signs of diarrhea and fecal samples were collected daily for virological analysis. Fecal samples were taken directly from the rectum by rectal stimulation and immediately transferred into sterile tubes and kept at 4°C. At the end of the experimental period on DPI4, small intestines were collected. The intestinal samples from each animal were placed in 10% buffered formalin saline, and remaining intestinal tissues were stored at -70°C for RT-qPCR. Animal studies were performed according to the guidelines for the care and use of laboratory animals issued by Sahmyook University (SYUIACUC 2013-007).

Light microscopy

Small intestinal tissues for histopathological analysis were processed using routine histology procedures and embedded in paraffin. Tissue sections (4-5 μm) were stained with haematoxylin and eosin and examined for possible histopathological changes under a high-resolution microscope equipped with a photographic facility.

Real-time quantitative PCR assay

RNA was extracted from stools and small intestines using the GeneAll® RibospinvRD™ kit (GeneAll, Korea) and the RNeasy® Mini kit (Qiagen, Germany), respectively. cDNAs of fecal specimens and intestinal tissues were synthesized using Oligo-dT primer and the Omniscript® Transcription kit (Qiagen). Quantification of cDNA was carried out by nano spectrophotometer at 260/280 nm.

Primers were designed to specifically amplify the VP7 gene of rotavirus by Bioneer (Bioneer, Korea). The primers VP7-F (sense, 5’-CTG ACG AAG CGA ATA AAT GG-3’) and VP7-R (antisense, 5’-GGT CAC ATC ATA CAA TTC T-3’) were used as forward and reverse primers, respectively. Beta-actin was used as internal control and its forward and reverse primer sequences were 5’-TGG AAT CCT GTG GCA-3’ and 3’-CCA GAT GAA CAA GGT TCA GTC-5’.
TCC ATG AAA C-3′ and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′, respectively.

RT-qPCR was conducted using the StepOnePlus™ RT-qPCR kit (Applied Biosystems, USA) and the Power SYBR® Green PCR Master Mix (Applied Biosystems, UK). Data were analyzed using StepOneTM/StepOneplus™ Software version 2.0. RT-qPCR was carried out in 20 μl reaction mixtures containing 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 μl of VP7 primer or 1.8 μl of β-actin primer, 2 μl of cDNA template, and 7.2 μl of dH2O or 4.4 μl of dH2O. The amplification conditions used were; initial incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and 72°C for 10 sec. At least three independent assays were performed. Relative quantifications of target gene were performed using the ΔΔCT method (Livak and Schmittgen, 2001).

Statistical analysis

Results were expressed as mean standard deviation (SD). Significant differences were separated using Duncan’s multiple range tests and commercial statistical analysis software, version 9.0 (SAS Institute, USA). All data were considered significant at P<0.05.

Results

Small intestine weights

On DPI 4, small intestine samples were collected and weighted. Small intestine weights in the PRO-1 and PRO-2 groups were greater than in the placebo and PBS groups. All intestine weights in the four groups were lower than in the normal animals. There was statistical significance between the PRO-1 and placebo groups. The intestinal weight of PBS group was decreased, but there was not statistical significance between the PRO-2 and PBS groups. Results were compared by calculating small intestine weight to body weight ratios (Fig. 1).

Light microscopic findings

H&E staining was performed on small intestinal samples from the four treatment groups and normal animals group. In infected rat, histopathological changes were characterized by vacuolization of the enterocytes, swelling of the villus tips, constriction of the bases, and nuclei that were irregularly positioned within the cells. Therefore, intestinal villi in the placebo and PBS groups were shortened and villous epithelial necrosis was observed, but these changes were not evident in the PRO-1 or 2 groups or normal animals group. Histopathological changes caused by rotavirus infection were characterized using cellular vacuolation which was observed in the basal regions of villi. The placebo and PBS groups exhibited more severe cellular vacuolation than the PRO-1 and 2 groups (Figs. 2 and 3).

Sensitivity and specificity of real–time quantitative PCR assay

To determine the effects of PRO-1 and 2 on rotavirus infection, RT-qPCR analysis was performed for the rotavirus VP7 gene. When comparing the CT values of fecal samples between the PRO-1 and placebo group, the VP7 gene was reduced by PRO-1.

Fig. 1. Small intestine weights. Results were presented as small intestine to animal weight ratios. The normal animals group were orally administered PBS instead of rotavirus inoculation. The PRO-1 and PRO-2 groups were inoculated with rotavirus, and orally administered PRO-1 or PRO-2 for 4 days from DPI 1. Animals inoculated with rotavirus were treated orally with placebo or PBS for 4 days from DPI 1. a,b Means with different superscripts differ.

Fig. 2. Morphological changes in small intestines. (A) Normal animal group, (B) PRO-1 group, (C) Placebo group as control. The normal animal group was orally administered PBS but not rotavirus or probiotic mixtures. Histopathological changes were vacuolization of the enterocytes (arrow), villous epithelial necrosis. Samples were H&E stained and observed under a light microscope. Original magnification×40.
Fig. 3. Morphological changes in small intestines. (A) Normal animal group, (B) PRO-2 group, (C) PBS group as control. The normal animal group was orally administered PBS but not rotavirus or probiotic mixtures. Histopathological changes were vacuolization of the enterocytes (arrow), villous epithelial necrosis. Samples were H&E stained and observed under a light microscope. Original magnification×40.

Likewise, the reduction of VP7 gene was observed in PRO-2 group (Fig. 4). Moreover, the PRO-1 and 2 groups showed that rotavirus mRNA transcript levels in small intestinal epithelial cells were lower than in the placebo or PBS groups (Fig. 5).

**Discussion**

Several studies have described the effects *Lactobacillus* and *Bifidobacteria* have on rotaviral gastrointestinal diseases (Majamaa et al., 1995; Cha et al., 1999; Bae et al., 2002; Ventola et al., 2012). *B. longum* maintains human gastrointestinal tract balance and is used as a probiotic in various dairy products (Lin and Chang, 2000; Šrutková et al., 2011). It improves lactose tolerance and has been used to diarrhea and food allergies (Schell et al., 2002; Yuan et al., 2006). In addition, *B. longum* has been reported to arteriosclerosis and stroke (Lin and Chang, 2000). And in a recent study, *B. lactis* was found to have a preventive effect on rotavirus infection (Shu et al., 2001). *L. acidophilus* has been reported to increase gastrointestinal tract resistance to bile, acid pH, and digestive enzymes (Collado et al., 2009). *L. rhamnosus* has been demonstrated to rotavirus diarrhea in children (Vanderhoof et al., 1999; Guandalini et al., 2000; Canaani et al., 2007), and *L. plantarum*, which is present in fermented food products, such as, Korean kimchi, helps...
antimicrobials survive in the human gastrointestinal tract (Nyborn et al., 2008; Bixquert Jiménnez, 2009; Bested et al., 2013).

In the present study, PRO-1 contained *B. longum*, *B. lactis*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, and *Pedioécoccus pentosaceus*, PRO-2 contained all PRO-1 components except *L. acidophilus*. According to clinical studies which used similar probiotics, compared to before and after treatment of probiotics, it shows that probiotics group were significantly increased in intestine (Yang et al., 2014; Yeun and Lee, 2014). Another studies show that different probiotics but, were similar results in the SD rats (An et al., 2011; Lee et al., 2011).

RT-qPCR assay uses VP7 genes to determine whether rotavirus has infected. VP7 genes are important for rotavirus replication, which starts when its RNA enters a host nucleus (Li et al., 2010). In this study, we investigated some of the effects of probiotic mixtures on rotavirus infection, but we were unable to determine the mechanisms involved. In addition, morphological changes of small intestines were not observed longitudinally. According to studies, some *Bifidobacterium* species are able to inhibit viral pathogen growth by blocking binding sites on epithelial cells (Colbère-Garapin et al., 2007; Dykstra et al., 2011). And another concluded, probiotics can improve the gut mucosal barrier, normalize intestinal permeability, and disrupt viral cycles using specific or non-specific mechanisms (Picard et al., 2005). The transcriptions of cellular genes, translation and post-translational mechanisms, protein stability, and the concentrations and distributions of second messengers can be affected by probiotics (Rosen et al., 2004). It has also been shown that the viral cycle is controlled by probiotics through signal translation pathways. One study reported that intestinal villi of infected rats were shortened maximally at 24 h post-infection, and in the syncytial cell formation in villi was observed at 18 hours post-infection in jejunum and at 24 h in ileum (Salim et al., 1995).

In this study results show that probiotic mixtures have the ability to inhibit rotavirus *in vivo* and to decrease the duration of rotavirus-induced diarrhea in rats. We suggest that these probiotic mixtures be considered for the treatment or as alternative therapies for rotaviral gastroenteritis. However, clinical trials are required to confirm that the effects observed in the present study occur in humans.

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References


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