보건학 석사학위논문

Anti-noroviral effects of *Lactobacillus* sp. isolated from Korean population

한국인에서 순수배양된 유산균의 항노로바이러스 효과 연구

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서울대학교 보건대학원

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ABSTRACT

Anti-noroviral effects of *Lactobacillus* sp. isolated from Korean population

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Norovirus gastroenteritis causes large health and economic costs, including at least 200,000 deaths annually. There is a difficulty of improve available vaccination program cause of limited cell culture system and effective treatment and prevention was not available until recently.

Probiotics is live bacteria have potential health benefits on the host. It is known as playing the role of immune modulation in the human

intestine. And previous studies said Probiotic bacteria have antiviral effects via various mechanism. In this study, we focused on the antiviral mechanism of the probiotics through activating innate immune response. Probiotics therapy could be an alternative method of antiviral prevention and modulation against norovirus infection.

In this study, we isolated target strains of *Lactobacillus* spp. successfully in the feces of Korean infants. And we screened the anti-noroviral activity of isolated *Lactobacillus* strains. *Lactobacillus reuteri* SNUG50382 (LR50382) significantly inhibited murine norovirus infection in RAW264.7 cells with having highest probiotics potential.

Both onset and resolution of disease symptoms are rapid, suggesting that components of the innate immune response are critical in norovirus control. To clarify the mechanism of norovirus inhibition of probiotics, we investigated gene expression of Interferon (IFN)-signaling components and IFN-inducible antiviral effectors. LR50382 increased IFN-α and IFN-β levels in RAW264.7 cells compared with the control. Gene expression of IFN regulating factor 7 (IRF7) and IFN-inducible antiviral effectors, Mx1 also increased. Overall, these results indicate that LR50382 efficiently inhibits norovirus replication *in vitro* due to its modulating capacity of the immune response through promoting type I IFNs, which are key regulators in IFN signaling pathway.

Keywords: Probiotics; *Lactobacillus*; antiviral effect; innate immune response; Norovirus; Isolation

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Ⅰ. Introduction

Human noroviruses (HuNoV) are enteric pathogens responsible for the majority of epidemic viral agent of acute gastroenteritis among all age groups worldwide. HuNoV causes approximately 90% of all epidemic nonbacterial outbreaks of gastroenteritis around the world, and in the United States alone, approximately 50% of all foodborne outbreaks caused by HuNoV and about 23 million people a year are infected with HuNoV where most outbreaks occur in day care settings, nursing homes, and cruise ships[1,2]. Viral infection causes various clinical symptoms, including explosive vomiting and diarrhea, nausea, abdominal pain, and low-grade fever lasting three to seven days. Unfortunately, there is no current treatment or vaccine effective against norovirus infection and little is known about the molecular mechanisms mediating HuNoV pathogenesis. The discovery of murine norovirus (MNV) and the establishment of an MNV animal model as well as a cell culture system have significantly broadened our understanding of the host and viral determinants of norovirus pathogenesis.

The gut microbiota plays a pivotal role in pathogen infection and mucosal immune responses through cross-talk with mucosal immune systems[3,4]. For example, an altered gut microbiome in mice lacking Toll-like receptors(TLRs) and myeloid differentiation primary response gene 88(Myd88) was strongly associated with metabolic syndrome, type 1 diabetes (T1D) and host defense against microbial infection[5-9].

Probiotics is live bacteria have potential health benefits on the host. Consumption of probiotics could help for maintaining homeostasis of microbiota in intestine, that means probiotics have relationship with immune system to an appropriate condition. It's thought to be important for the prevention of infectious disease caused by external pathogens[10].

In the previous research, the fecal microbial composition was different between the viral diarrhea groups and healthy group[11]. In viral diarrhea, copy number of lactobacilli and *Bifodobacteria* in feces was reduced which means intestinal microbiota composition have effect on viral infection especially the species like *Lactobacillus* and *Bifodobacteria*. Besides there were more specific studies about antiviral effects of lactobacilli. Y. Nakayama said the oral administration of *L. gasseri* SBT2055 exhibits efficacy to protect mice infected with the influenza virus A/PR8 by induction of antiviral genes expression[12]. And J.Y. Kang demonstrated the isolated probiotics including *L. ruminis* SPM0211 have effects of inhibition to rotavirus replication in a rotavirus-infected neonatal mouse model by increasing gene expression of IFN-inducible antiviral effectors[13].

In this study, we isolated one of the gut microbiota, *Lactobacillus* strains from fresh feces of Korean infants and evaluated the anti-noroviral effects of *Lactobacillus* strains. The MNV titer was significantly decreased in MNV-infected Raw264.7 cells incubated with live *Lactobacillus* strains and then we characterized the probiotic potential of isolated lactobacilli by using *in vitro* evaluation methods.

One isolate, identified as *Lactobacillus reuteri*, The *L. reuteri* SNUG50382

(LR50382) is a probiotic lactic acid bacterium isolated from Korean infants feces with properties such as acid, bile tolerance, and to improve the intestinal environment with shown low resistance to antibiotics.

To clarify antiviral effects of LR50382, Immunomodulatory function of LR50382 demonstrated through MNV-infected Raw264.7 cells. We identified The treatment of LR50382 to MNV-infected Raw264.7 cells induced the expression of the antiviral gene (Interferon-α, Interferon-β), Interferon regulatory factor 3/7, and myxovirus resistance 1 (Mx1) mRNAs. Especially Interferon-β (IFN-β) significantly increased in LR50382 treated-RAW264.7 cells even though without MNV. These results indicate that the administration of LR50382 is efficient for the prevention of MNV by the inhibition of virus replication via up-regulation of the expression of antiviral genes such as IFN-β.

Ⅱ. Materials and Methods

2-1. Description of study subjects and fecal samples

All participants provided written informed consent. Fecal samples came from infants who visit to the hospital for a check-up. A total of 20 samples were collected within 12hours by Samsung Medical Center, Seoul in Korea. Subjects were aged under 12 months. As soon as sampling, fresh fecal was transported to the laboratory with ice-pack for retaining composition of bacteria and viability. Each fecal samples was stored in deep freezer at -80℃. The study protocol was approved by the Korea Centers for Disease Control and the institutional review board (IRB) of the Samsung Medical Center.

2-2. Culturing of fecal microbiota

Fresh fecal sample was spread directly onto selective media using disposal spreader (SPL, USA) in the anaerobic chamber (COY). The culture media used in this study were BL agar with Tween 80 (KFDA, Kisan Biotech.), BSM agar (Bifidus Selective Medium Agar, Fluka), and TOS-propionate agar (Transgalctosylated oligosaccharide agar medium, Sigma). After 48h incubation in anaerobic condition at 37℃, we performed colony picking and streaking it in BL agar, BSM agar, and TOS agar for isolation single colony. Colonies were cultured for 24hours at 37℃ in anaerobic condition. Pure colonies were then selected by loop & needle 1 μl (SPL, USA) and enriched

in 1.5mL MRS broth into 96 deep well plates at 37℃ under anaerobic condition[14].

2-3. Bacteria identification

Bacteria pellet was collected by centrifugation. After washing with 1 × phosphate-buffered saline (PBS), 0.2 μl of pellet in lysis buffer was boiled at 95℃ for 10minutes.

Of each samples, 16S rRNA regions were amplified using 27F/1492R primer set. (27F: forward primer, 5' – AGA GTT TGA TCM TGG CTC AG – 3'; 1492R: reverse primer, 5' – GGY TAC CTT CTT ACG ACT T – 3'). Amplified PCR products were purified using QIAquick PCR Purification kit (Qiagen, Germany). The identification of Bacterial 16S rRNA sequence was used by EZtaxon in EZbioCloud (Jongsik Chun's laboratory at Seoul National University and ChunLab, Inc.)[14].

2-4. Cells and viruses

The murine leukemia tumor macrophage cell line, RAW 264.7 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum (Gibco), 10mM HEPES, 10mM sodium bicarbonate, 10mM non-essential amino acids, and 50 μg/mL gentamicin at 37℃ in 5% CO_2 air atmosphere. For virus infection, the culture media was changed into media with 2% fetal bovine serum.

2-5. In vitro assay to examine MNV inhibition by *Lactobacillus* spp.

All Lactobacillus strains from microbial isolates from feces of infants were cultured in MRS broth at 37℃ for 24hours and sub cultured in fresh growth medium for 18hours. Prior to the inhibition assay, grown bacteria were harvested by centrifugation 13,000rpm for 5 minute and washed two times with 1 × PBS. Quantity of bacteria was estimated using optical density(OD). Several experiment indicated that culture media measured 0.1 OD means harboring approximately 1 × 108 CFU/ml concentration of bacteria. A cell viability test was performed and the concentration of the antibiotics (ampicillin 50 μg/mL) was determined, which represents the steady state of Lactobacillus.

MNV CW1 (MOI 0.01) was infected into RAW 264.7 cells for 1 hour. Following a media change, *Lactobacillus* estimated by OD was inoculated for 24 h in MNV-infected RAW264.7 cells. After 24 h, cells were performed a plaque assay to quantify MNV after freezing and thawing twice[15].

2-6. Test of *Lactobacillus* spp. for acid-tolerance

Each strain of *Lactobacillus* isolates was grown once in MRS broth from the stock freezer vial for 18 to 24 hour at 37℃ before use in an experiment. After activation of bacteria, inoculated (1%) into MRS broth acidified with concentrated hydrochloric acid to pH 4, pH 3 and pH 2. After incubating at 37℃ under anaerobic conditions, Absorbance of the culture media was measured

with 600nm by optical density(OD). Strains that showed little reduction against optical density of growth media untreated acid (pH 6.8) were considered to be candidates for selection of acid-tolerant strains.

2-7. Test of *Lactobacillus* spp. for bile salts-tolerance

Cultures were grown in MRS broth containing 0.05% cysteine. After restoration of bacteria activity, the survival of lactobacilli strains was examined in MRS broth containing bile salts concentrations 1%, 2%, and 4% w/v. Each strain was inoculated (1% v/v) into different three conditions and experiment with a third time. Absorbance 600nm was measured and the results were expressed as the percentage of growth (A600 nm) in the presence of bile salts compared to the control (without bile salts).

2-8. Antibiotic susceptibility

For selecting *Lactobacillus* strains, the MICs (μg/mL) of nine antibiotics were determined using commercial E-test ® (Epsilometer test, bioMerieux, France): ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. The concentration on the strips was from 0.016 to 256 μg/mL with the exception of streptomycin (0.064-1024 μg/mL). Bacterial cultures in the exponential growth phase were diluted to a suitable turbidity and used to inoculate a melted and cooled iso-sensitest agar (90% w/v, Oxoid, UK) supplemented with MRS agar (10% w/v) to a final concentration of ~106-107 CFU/mL. E-

test® strips were placed on the surface of the inoculated agar and incubated at 37℃ for 24h. The MIC was interpreted as the point at which the ellipse intersected the E-test® strip as described in the E-test technical guide[16].

2-9. Cytotoxicity assay

The cytotoxicity of the probiotic bacterial strains on cultured cells was assessed using the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertrazolium bromide] (Sigma) assay. Raw264.7 cell were seeded on 96-well plates at a density of 1×10^4cells/well with 1×10^6 CFU/ml concentration estimated by OD of *Lactobacillus* spp. and incubated for 24 h. Cells with media alone were used as the control group. The incubation medium was removed and 100 μl of MTT solution was added to each well. After incubation for 4 h at 37℃, The MTT solution was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. Viable cells were detected by measuring absorbance at 540 nm.

2-10. Quantitative RT-PCR

Total RNA from cells was isolated using easy-spin RNA extraction Kit (Intron bio). cDNA was then synthesized from 1 μg of total RNA using High Capacity RNA-to-cDNA kit (applied biosystems, Thermo FS). The levels of MNV genomes copies, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and other cytokines were determined by qRT-PCR. The reaction mixture (25 μL) for real-time PCR was composed of 2x RG SYBR PCR

master Mix (12.5 μL), primers (IFN-α4: forward and reverse, IFN-β: forward ATG AAC AAC AGG TGG ATC CTC C and reverse AGG AGC TCC TGA CAT TTC CGA A, IRF7: forward and reverse, and Mx1: TCT GAG GAG AGC CAG ACG AT and reverse ACT CTG GTC CCC AAT GAC AG, each 50 pmol in 0.5 μL), RNase-free water (10.5 μL), and template DNA (1 μL). GAPDH was used as the internal control. MNV copy number was determined by comparing Ct values to a standard curve generated by dilution of a plasmid encoding the target sequence of interest. And the levels of expression of other genes was calculated by comparing Ct values to housekeeping gene's Ct values[17]. Each sample was analyzed in three times.

2-11. Statistical analysis

The results acquired form this study were presented as mean ± standard error of mean (SEM) or mean ± standard deviation of mean (SD). The statistical significance of differences was analyzed using R statistics for window ver. 3.3.0 (Lucent Tech, USA) and GraphPad Prism (GraphPad software version 5). One-way ANOVA and student's *t* test was used for statistical analyses. All statistical tests were evaluated at the 95% confidence level. n.s. = $p>0.05$, *= $p\leq0.05$, **= $p\leq0.01$ ***= $p\leq0.001$.

Ⅲ. Results

3-1. Isolation of *Lactobacillus* strains and cell viability

We isolated total 580 strains of enteric bacteria including 47 *Lactobacillus* strains. Isolated *Lactobacillus* spp. suitable to below conditions were regarded as same bacteria; same fecal sample, same selective media, and identified as same strains. Fifteen *Lactobacillus* strains were selected from 47 *Lactobacillus* strains. Feces origin information of selected bacteria was descripted in Table 1. We investigated cytotoxicity of selected strains by MTT assay (Table 1). Raw264.7 cell viability after treatment with live bacteria for 24 hours ranged from 68.46% to 100% compared with control group. *Lactobacillus rhamnosus* SNUG50057, 50070, 50362, and *Lactobacillus gasseri* SNUG50019 showed cell viability percentage below 80. These strains are considered having cytotoxicity in some degree, which could influence reliability of inhibitory activity on MNV.

Table 1. Effects of probiotics isolated from Korean infants on the viability in Raw264.7 cells

Species	Origin		Cell viability (% of control)[a]
	Sex	Age(month)	
Lactobacillus rhamnosus			
SNUG50057	Female	2	75.96 ± 9.74
SNUG50070	Female	2	75.17 ± 7.76
SNUG50362	Female	2	74.40 ± 8.49
SNUG50415	Female	4	94.01 ± 4.82
SNUG50461	Female	4	99.13 ± 9.36
SNUG50545	Female	3	98.52 ± 3.93
Lactobacillus gasseri			
SNUG50019	Female	4	68.46 ± 6.49
SNUG50077	Female	2	93.15 ± 6.05
SNUG50243	Female	4	81.79 ± 5.94
SNUG50390	Female	2	99.93 ± 6.17
SNUG50417	Female	4	98.11 ± 8.70
SNUG50579	Male	3	82.79 ± 6.98
Lactobacillus reuteri			
SNUG50382	Female	2	93.79 ± 6.44
Lactobacillus casei			
SNUG50500	Male	3	100.00 ± 8.45
Lactobacillus paracasei			
SNUG50501	Male	3	90.12 ± 10.07

[a] The value represents the mean ± SEM

3-2. Inhibition of MNV replication by *Lactobacillus* strains *in vitro*

MNV-infected RAW264.7 cells were treated fifteen selected *Lactobacillus* strains in each well. After 24 hours, MNV titer were measured by plaque assays on Raw264.7 cells to select the probiotic strain showing the more effective inhibitory effects. all selected *Lactobacillus* strains showed a considerable inhibitory effect on MNV replication compared to MNV infection alone (10^{-2} to $10^{-3.9}$, Fig. 1). However, these results clearly showed strain specific-inhibitory effect. Even if they were classified under same species, *Lactobacillus gasseri* SNUG50579 decreased MNV titer to 2 log, meanwhile *Lactobacillus rhamnosus* SNUG 50070 showed almost 4 log reduction.

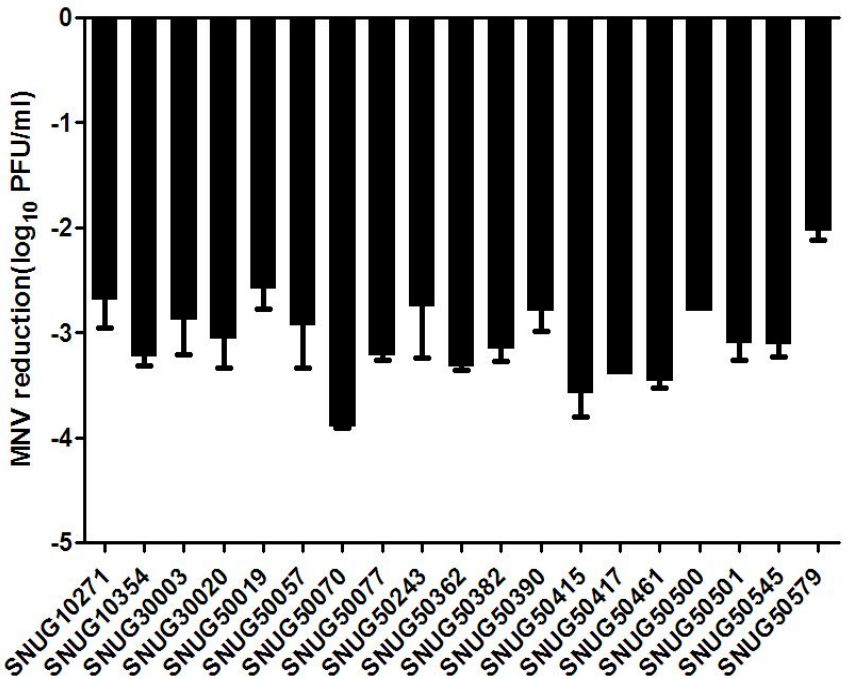

Figure 1. Antiviral effects of *Lactobacillus* spp. isolated from Korean infants on MNV- infected Raw264.7 cells

Raw264.7 cells (1 x 10^6 cells/well) were infected with MNV(MOI0.01) for 1h and treated with Lactobacillus (estimated to 1 x 10^8 CFU by OD) for 24h. Antiviral activity was determined by plaque assay. Values shown represents the mean ±SD.

3-3. Screening of acid-tolerance of *Lactobacillus* isolates

Because the pH state of human gastrointestinal track altered to highly acidic, candidates of probiotics are required to have tolerance in acidic environment.

We investigated the viability of 15 lactobacilli elected from 47 isolates in pH 2,3,4 MRS broth. The percentage of growth rate in pH 2,3,4 MRS broth calculated against growth rate in standard MRS medium (pH initial 6.7). The pH4 MRS broth gave higher viability of *Lactobacillus* isolates than did pH3 or pH2 MRS broth (Table 2). As the pH of the growth medium lowered to 2, the survival rates of most bacteria were decreased in below 10%. However, in culture media pH 4, most strains remained half viable or more and exhibited significant growth rates. Especially strain *Lactobacillus reuteri* SNUG50382 (LR50382) showed the highest growth level in pH4 culture medium staying even over 100% growth rate.

3-4. Screening of bile salts-tolerance of *Lactobacillus* isolates

Tolerance to bile salts is important for the probiotic strains to grow and survive in the human intestine. In this study, the survival of *Lactobacillus* strains was examined in MRS broth containing bile salts concentrations 1,2, and 4%.

Results for the ability to endure bile salt from 15 strains were more or less

resistant to 2% bile salts (Table 3).

Judging by the result, LR50382 could grow well in MRS media including high percentage of bile salts (4%) than others, as same as showing the highest tolerance rate in acid-tolerance test.

Table 2. Acid-tolerance of tested *Lactobacillus* strains (pH 4, 3, 2)

Tested strain	Viability (% of control)		
	pH 4	pH 3	pH 2
Lactobacillus rhamnosus SNUG50057	68.22 ±21.54	28.07 ±12.90	3.71 ±0.34
Lactobacillus rhamnosus SNUG50070	47.18 ±6.70	5.41 ±0.96	2.98 ±0.09
Lactobacillus rhamnosus SNUG50362	49.96 ±4.61	4.54 ±0.90	2.38 ±0.05
Lactobacillus rhamnosus SNUG50415	45.83 ±4.21	4.36 ±1.73	2.44 ±0.27
Lactobacillus rhamnosus SNUG50461	28.11 ±4.84	4.71 ±1.10	4.84 ±2.20
Lactobacillus rhamnosus SNUG50545	48.26 ±7.18	4.40 ±1.49	2.45 ±0.30
Lactobacillus gasseri SNUG50019	52.70 ±5.61	6.01 ±1.31	3.68 ±1.61
Lactobacillus gasseri SNUG50077	49.00 ±8.32	7.46 ±1.15	4.76 ±2.53
Lactobacillus gasseri SNUG50243	51.93 ±5.84	3.77 ±1.25	2.00 ±1.65
Lactobacillus gasseri SNUG50390	48.33 ±6.07	4.58 ±0.10	2.84 ±1.53
Lactobacillus gasseri SNUG50417	26.31 ±0.73	2.01 ±1.19	7.03 ±14.09
Lactobacillus gasseri SNUG50579	46.79 ±3.94	2.98 ±0.13	3.81 ±1.67
Lactobacillus reuteri SNUG50382	105.22 ±4.23	4.99 ±1.87	1.26 ±1.13
Lactobacillus casei SNUG50500	50.62 ±4.63	3.25 ±1.71	1.86 ±2.10
Lactobacillus paracasei subsp. tolerans SNUG50501	46.58 ±11.19	2.96 ±1.31	2.19 ±0.80

Table 3. Bile-tolerance of tested *Lactobacillus* strains (bile salt 1, 2, 4%)

Tested strain	Viability (% of control)		
	Bile salts 1%	**Bile salts 2%**	**Bile salts 4%**
Lactobacillus rhamnosus SNUG50057	29.52 ± 17.03	24.39 ± 16.55	22.88 ± 3.65
Lactobacillus rhamnosus SNUG50070	17.35 ± 3.25	11.01 ± 3.16	21.53 ± 5.09
Lactobacillus rhamnosus SNUG50362	20.62 ± 4.93	17.52 ± 5.95	22.07 ± 6.88
Lactobacillus rhamnosus SNUG50415	15.42 ± 6.17	10.77 ± 4.08	20.02 ± 7.67
Lactobacillus rhamnosus SNUG50461	1.02 ± 0.08	1.05 ± 0.15	6.43 ± 3.39
Lactobacillus rhamnosus SNUG50545	17.45 ± 1.34	18.26 ± 9.91	22.89 ± 4.72
Lactobacillus gasseri SNUG50019	0.50 ± 0.61	0.62 ± 0.18	5.22 ± 7.51
Lactobacillus gasseri SNUG50077	0.21 ± 0.07	0.63 ± 0.20	21.14 ± 1.74
Lactobacillus gasseri SNUG50243	2.14 ± 0.21	2.81 ± 0.85	2.34 ± 4.31
Lactobacillus gasseri SNUG50390	0.71 ± 0.03	2.20 ± 1.52	5.02 ± 0.97
Lactobacillus gasseri SNUG50417	1.47 ± 1.21	1.09 ± 1.02	6.64 ± 11.08
Lactobacillus gasseri SNUG50579	0.03 ± 1.36	0.42 ± 0.24	9.42 ± 1.87
Lactobacillus reuteri SNUG50382	55.88 ± 23.44	46.32 ± 13.64	29.07 ± 2.59
Lactobacillus casei SNUG50500	7.24 ± 5.19	3.10 ± 3.65	6.22 ± 2.79
Lactobacillus paracasei subsp. tolerans SNUG50501	10.87 ± 4.72	8.04 ± 4.91	8.41 ± 2.18

3-5. The test of the antibiotic susceptibility of the LR50382

We narrowed down experimental strains to one outstanding isolated strain with reference of acid, bile tolerance. LR50382 showed highest acid, bile-tolerance survival rate as presented above. And we selected *Lactobacillus rhamnosus* SNUG50070 too because of topmost inhibitory effects on MNV replication.

In addition to acid, bile-tolerance test, the MICs of nine antimicrobials of human importance were determined for the elected isolate. Lack of transferable resistance against therapeutic antibiotics is an important criterion for selection of an appropriate functional strain. Two groups of antibiotics are generally recommended: inhibitors of cell-wall synthesis (ampicillin and vancomycin) and inhibitors of protein synthesis (chloramphenicol, gentamicin, streptomycin, kanamycin, tetracycline, erythromycin and clindamycin).

The obtained results and reference microbiological breakpoints are presented in Table 4. A micro-organism inhibited at breakpoint level to a specific antimicrobial is defines as susceptible. When the MIC is higher than the breakpoint, the micro-organism is considered resistant. For the analysis, E-test® was chosen in this study, as it is a simple quantitative method that is commonly used for antimicrobial susceptibility testing of different micro-organisms.

LR50382 was susceptible toward ampicillin, erythromycin, kanamycin,

tetracycline, streptomycin and gentamicin and could be considered resistant to clindamycin and chloramphenicol with MICs higher than the breakpoints proposed by the FEEDAP Panel[18].

By contrast, *L. rhamnosus* SNUG50070 (LRh 50070) was resistant to most of antibiotics except chloramphenicol, erythromycin and gentamicin. It may be explained by the high rate of spontaneous chromosomal mutations conveying resistance to these antibiotics. So strain with lower MICs for antibiotics may be suitable to using as probiotics.

Growth rate is also important to determine possibility of probiotics. Figure 2 showed growth curve of LR50382 and LRh50070 in optical density values. Their exponential time is from 3 hours to 18 hours. We identified LR50382 grow faster than LRh50070, which is beneficial ability to probiotics.

Table 4. MIC (µg/mL) of antimicrobials for *Lactobacillus* strains

Tested strain	Susceptibility to the following antibiotic MIC (µg/mL)								
	AMP	**C**	**CD**	**E**	**VA**	**K**	**TE**	**S**	**CN**
EFSA J. *Lactobacillus reuteri*	**2**	**4**	**1**	**1**	**n.r.**	**64**	**16**	**64**	**8**
SNUG50382	0.75	6	1.5	0.25	>256	48	0.38	4	2
EFSA J. *Lactobacillus rhamnosus*	**4**	**4**	**1**	**1**	**n.r.**	**64**	**4**	**32**	**16**
SNUG50070	32	2	1	0.38	>256	96	16	64	4
SPM1308	0.016	2	0.25	1	>256	96	24	12	6

Note: AMP – ampicillin, C – chloramphenicol, CD – clindamycin, E – erythromycin, VA – vancomycin, K – kanamycin, TE – tetracycline, S – streptomycin, CN – gentamicin. n.r. – not required.
The bold values are reference breakpoints given by EFSA[18]. And that is they are visually emphasized. This would allow easier comparison with the values obtained for the tested strains.

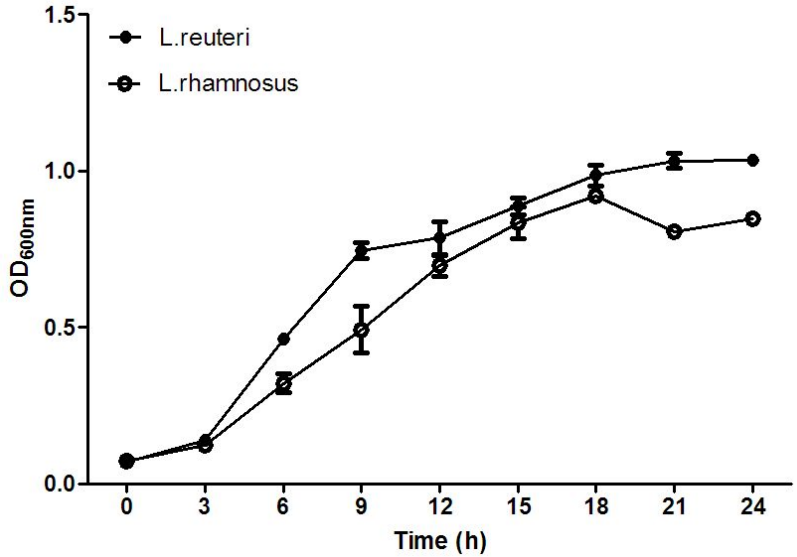

Figure 2. Growth curve of *L.reuteri* SNUG50382 and *L.rhamnosus* SNUG50070

Strains cultured in MRS broth with the collecting times marked. Optical density at 600 nm was used to assess density of growth. Values shown represents the mean ±SEM.

3-6. Antiviral activity of LR50382 on Raw264.7 cells

We carried out plaque assays with MNV-infected Raw 264.7 cells treated LR50382 24 hours or 48 hours using either 1×10^7 CFU/ml or 1×10^8 CFU/ml concentration. LR50382 demonstrated significant antiviral activity in MNV-infected Raw264.7 cells (Fig. 3A). LR50382 at 1×10^7 CFU/ml dropped plaques by 1.6 ~ 1.9 log scale compared to the control, and LR50382 at 1×10^8 CFU/ml decreased plaque formation a further 2.4 ~ 3.1 log scale, indicating that LR50382 dose-dependently reduced the plaque formation on Raw264.7 cells.

To additional evidence of antiviral activity of LR50382, we performed plaque assays with different incubation time. LR50382 showed significant decrease in MNV plaque forming units in both incubation time conditions (Fig. 3B). In 24 hours incubation, LR50382 decreased plaque formation to 1.7 ~ 2 log scale. And after 48 hours culture, although MNV PFU proliferated 10 times than PFU of 24h incubation because incubation time become longer, LR50382 also dropped plaque formation by 1.7 ~ 1.9 log scale as similar as plaque formation reduction rates of 24h incubation.

To determine whether the LR50382 had antiviral activity through inhibition of murine norovirus replication, we evaluated MNV genome expression in MNV-infected Raw264.7 cells by RT-qPCR (Fig. 4). The genome copies of MNV significantly decreased when 10^7 CFU/ml LR50382 was applied to MNV infected-RAW264.7 cells for 24 hours comparing to MNV-infected cells (p=0.0048). And after 48 hours incubation, the MNV genome declined

almost 10 times more than that of 24 h incubation comparing to positive control (p=0.0047). when MNV infected RAW264.7 cells were incubated with LR50382 of 10^8 CFU/ml concentration for 24 h, 48 h, MNV genome copies were reduced significantly as well (p=0.01, p=0.005).

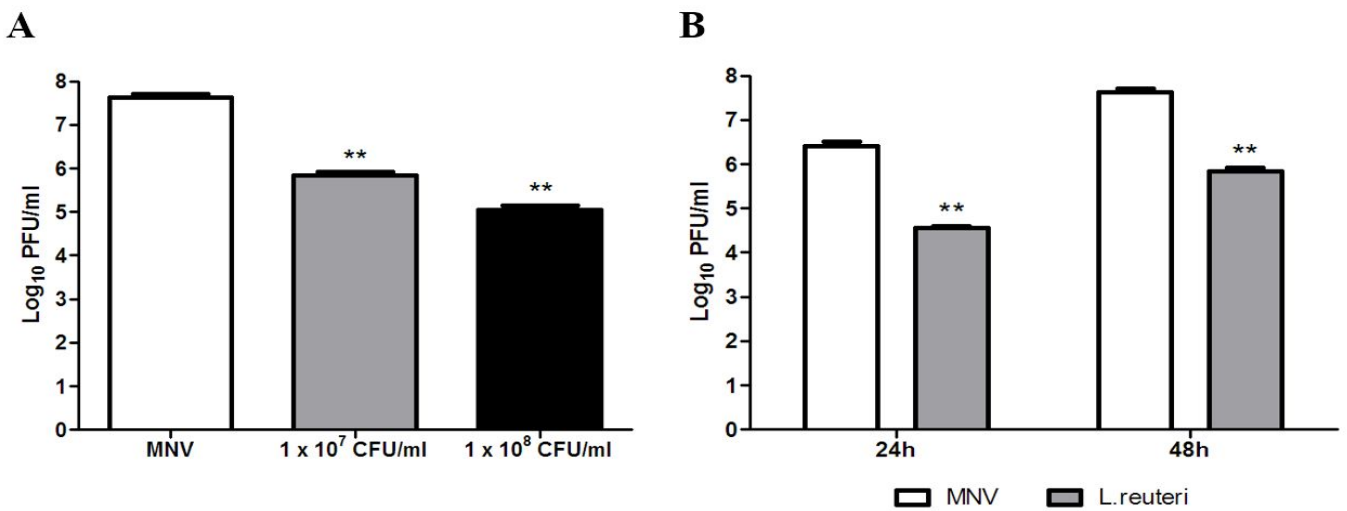

Figure 3. Antiviral effects of *Lactobacillus reuteri* SNUG50382 on MNV- infected Raw264.7 cells

Raw264.7 cells (1 x 10^6 cells/ml) were infected with MNV (MOI0.01) for 1h and treated with live *L. reuteri* SNUG50382 (estimated to 1 x 10^7 CFU/ml, 1 x 10^8 CFU/ml by OD) for 24 h, 48 h each. Antiviral activity was determined by plaque assay. We repeated the independent experiment at least four times. Values shown represents the mean ±SEM. Significant differences relative to positive control (MNV-infected Raw264.7 cells), *P<0.05, **P<0.01

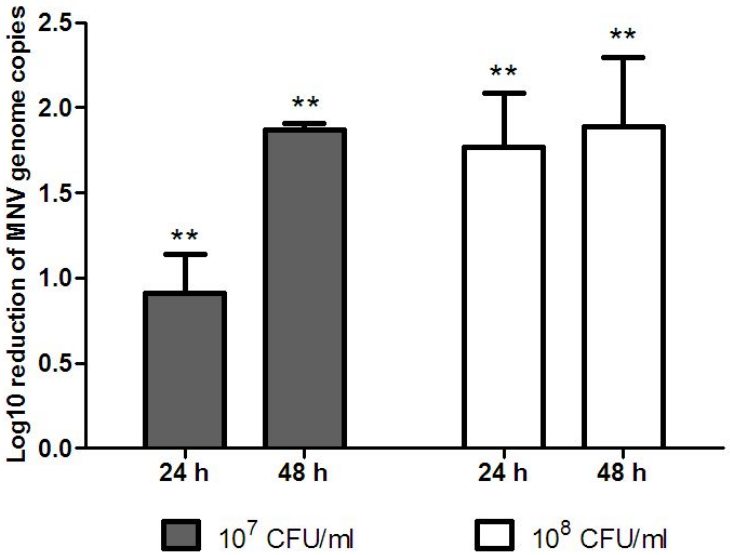

Figure 4. Effects of *Lactobacillus reuteri* SNUG50382 on MNV gene expression in MNV- infected Raw264.7 cells

Raw264.7 cells (1 x 10^6 cells/ml) were infected with MNV (MOI0.01) for 1h and treated with live *L.reuteri* SNUG50382 (estimated to 1 x 10^7 CFU/ml, 1 x 10^8 CFU/ml by OD) for 24 h, 48 h each. Viral genome was determined by RT-qPCR. We repeated the independent experiment at least three times. Values shown represents the mean ±SEM. Significant differences relative to positive control (MNV-infected Raw264.7 cells), **P*<0.05, ***P*<0.01

3-7. Mechanism of antiviral activity of LR50382

We examined whether the *Lactobacillus* probiotics inhibited murine norovirus infection through stimulating a type Ⅰ IFN signaling pathway, one of the innate immune responses, in MNV-infected Raw264.7 cells by RT-qPCR. We observed that MNV infected-RAW264.7 cells with treated LR50382 showed high values of IFN-α and IFN-β gene expression comparing to non-infected cells (Fig. 5). Though the gene expression of treated cells incubated for 24 h showed little increase comparing to negative control (1.40, 1.59 Fold), the IFN-α gene expression level of treated cells incubated for 48 h increased 2.59, 3.61 fold values than negative control (Fig. 5A). IFN-β gene expression were increased four times more than control and steadily increased through time as well as dose dependently except 10^8 CFU/ml, 48-hour incubation (Fig. 5B).

We then measured Interferon regulatory factor 3/7, IRF3/7 and Mx1, which is IFN-inducible antiviral effectors (Fig. 6). IRF3/7 are transcription factors stimulated by phosphorylation through antiviral cascade pathways such as IKKε/TBK1 pathway. According to our results, IRF3 mRNA expression generally reduced in 24 hours incubated groups (Fig. 6A). But after 48 hours incubation, IRF3 expression level a little higher in infected-cells treated LR50382 dose-dependently than level of negative control. It is thought IRF3 is stimulated by TLR3, receptor recognizing dsRNA of virus, not ssRNA. In contrast, IRF7 is known as directed products by TLR7/8 recognizing ssRNA. And IRF7 is also one of the Interferon stimulated genes. So IRF7 expressed

almost 7 fold higher in treated groups than negative control expression (Fig.6B). And the IRF7 mRNA expression levels of All treated groups exceeded 2 times over than negative group.

We evaluated genomic expression level of Mx1, one of the Interferon stimulated genes in MNV-infected RAW264.7 cells. There is significant inducing Mx1 gene expression in Raw264.7 cells treated with 10^7 CFU/ml LR50382 for 48 h (Fig. 6C).

To confirm the immunomodulation ability of LR50382, we performed treatment on Raw264.7 cells with only live bacteria for 24 hours (Fig. 7). The gene expressions of IFN-β, IRF7, and Mx1 were significantly increased in Raw264.7 cells only treated with LR50382.

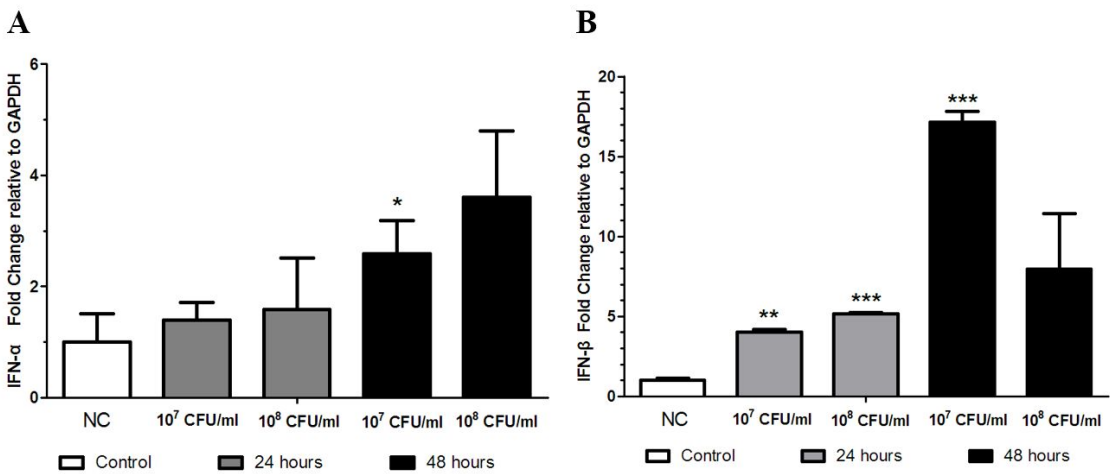

Figure 5. Effects of *Lactobacillus reuteri* SNUG50382 on MNV induced IFN-α (A) and IFN-β (B) gene expression in MNV-infected Raw264.7 cells.

Raw264.7 cells (1 x 10^6 cells/ml) were infected with MNV (MOI0.01) for 1h and treated with live *L. reuteri* SNUG50382 (estimated to 1 x 10^7 CFU/ml, 1 x 10^8 CFU/ml by OD) for 24 h, 48 h each. mRNA was determined by RT-PCR. We repeated the independent experiment at least three times. Values shown represents the mean ±SD. Significant differences comparing to non-infected cell (NC), **P*<0.05, ***P*<0.01, ****P*<0.001

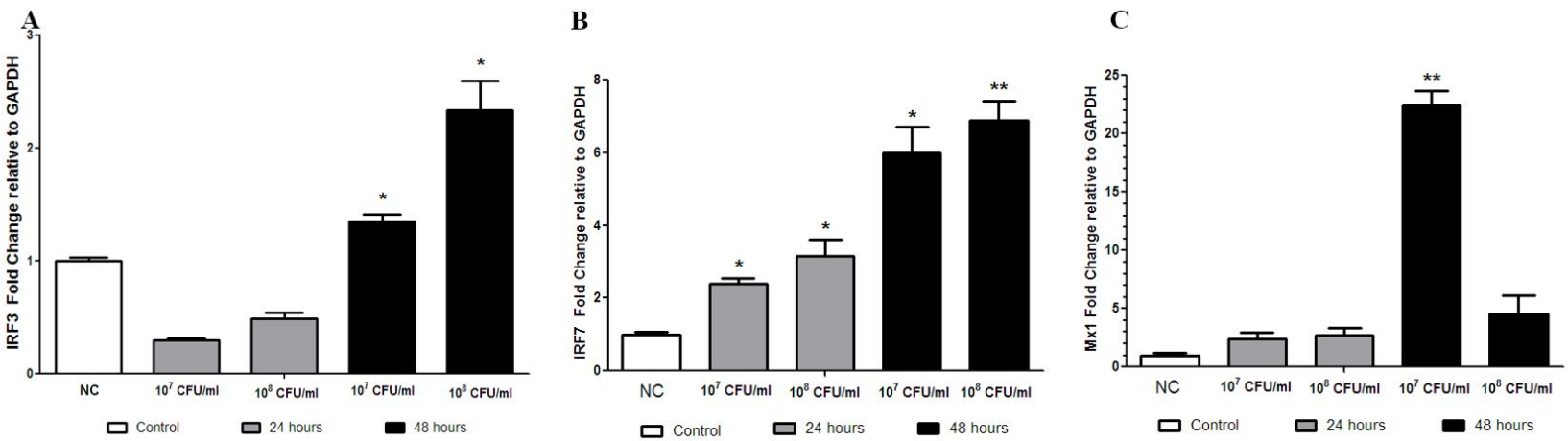

Figure 6. Effects of *L. reuteri* SNUG50382 on gene expression levels of IRF3 (A), IRF7 (B) and Mx1 (C) in MNV-infected Raw264.7 cells.

Raw264.7 cells (1 x 10^6 cells/ml) were infected with MNV (MOI0.01) for 1h and treated with live *L. reuteri* SNUG50382 (estimated to 1 x 10^7 CFU/ml, 1 x 10^8 CFU/ml by OD) for 24 h, 48 h each. mRNA was determined by RT-PCR. We repeated the independent experiment at

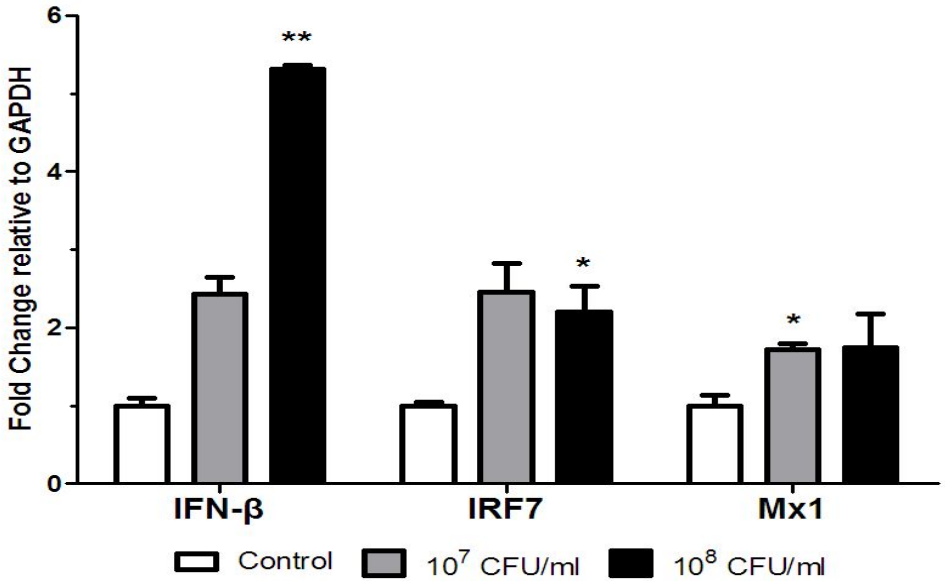

Figure 7. Effects of *Lactobacillus reuteri* SNUG50382 on gene expression levels of IFN-β, IRF7, and Mx1 in Raw264.7 cells

Raw264.7 cells (1 x 10^6 cells/ml) were seeded and treated with live *L. reuteri* SNUG50382 (estimated to 1 x 10^7 CFU/ml, 1 x 10^8 CFU/ml by OD) for 24 h. mRNA was determined by RT-PCR. Values shown represents the mean ±SEM. Significant differences, **P*<0.05,

IV. Discussion

The microbial ecology of the gastrointestinal tract includes the diversity of taxa, their activities, and relationship with each other and the host such as synergistic and competitive interactions[19]. Bacteria present in GI tract are a crucial line of defense against the colonization and tissue invasion by exogenous microbes or pathogens. Administration of probiotics is effective as an alternative treatment for moderating norovirus gastroenteritis as a bacteriotherapy. Guandalini and Foye et al. reported that probiotic bacteriotherapy is increasingly recognized to moderate infectious diarrhea and a mechanism to improve intestinal homeostasis[20,21]. And a number of pediatric clinical trials were reported that *L.acidophilus*, *L.paracasei*, *L.rhamnosus* GG, *L.reuteri*, *S. thermophiles*, and the probiotic mixture have significant effects on the treatment of diarrhea in children[22]. These studies clearly show that probiotics and its metabolites are good alternatives as low cost prevention and treatment agents.

In this study, we isolated fastidious target strains of *Lactobacillus* spp. successfully in the feces of Korean infants. And we screened the antiviral effects of Lactobacillus spp. against MNV replication in infected Raw264.7 cells. We selected probiotic bacteria *L.reuteri* SNUG50382 that showed the effective inhibitory ability to murine norovirus based on the results of a plaque assay and RT-qPCR. We identified decrease of MNV titer and genome in LR50382 treated-RAW264.7 cells dose-time dependently. The prevention

of norovirus infection through dietary supplementation has been considered important, as no effective treatment or vaccine against norovirus infection is currently available. This study provides glimpse of idea regarding the particular microorganisms in the gut, such as *L. reuteri* SNUG50382, as preventing acute viral gastroenteritis.

According to Colbere-Garapin probiotics could block viral attachment at the surface of intestinal cells by competitive inhibition during entry[23]. However, these results did not confirm whether the probiotics inhibited murine norovirus replication through modulation of the viral replication. Therefore, we evaluated murine norovirus RNA expression by treating MNV-infected RAW264.7 cells with selected probiotics. It demonstrated antiviral activity of probiotics inhibit MNV replication at genomic level. LR50382 dropped MNV gene expression by 1.0 – 2.5 log compared to control group dose-time dependently. Taken together, these results revealed that LR50382 inhibited MNV replication and infection *in vitro*.

Indeed, *Lactobacillu*s species produce lactic acids, which becomes lower pH values of culture media. pH values could have important role to virus survival ability in terms of inhibiting enzyme activity, structural stability, and packaging. For examples, Influenza A virus (IAVs) acquired Low-pH-stable ability showed much higher virus replication rates than those of low-pH-unstable IAVs[24]. But Murine norovirus reportedly persists over a 20-day period in a fermented vegetable product with a final pH below 4.0[25].

Components of the innate immune response are known as having critical role in norovirus Control[26]. Toll-like receptors (TLRs) are receptors that act as

the primary sensors of pathogen-associated molecular patterns (PAMPs) included innate immune recognition receptors family[27]. It is known that TLRs, especially endosomal TLRs, TLR2, -3, -4, -7, and -8 are thought to be crucial in the recognition of structural components of RNA viruses. Though all of TLR3, -7, and 8 recognizes nucleic acid motifs of RNA viruses, TLR3 mainly recognizes dsRNA, which constitutes the genome of some RNA Viruses like rotavirus, otherwise TLR7 and phylogenetically closely related to TLR7, TLR8 recognize ssRNA, the genome of some viruses including murine norovirus.

A common feature of all TLR recognition is the activation of three major signaling pathways: mitogen-activated protein kinases (MAPKs)p38 and JNK (c-Jun Kinase), transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and one or more interferon regulatory factors (IRFs). In certain situations, IRF3 and/or IRF7 are essential for induction of type Ⅰ IFNs because the others also activate inflammatory response and adaptive immune activator like tumor necrosis factor (TNF)[28]. with IRF3/7 are transcription factors stimulated by phosphorylation through antiviral cascade pathways such as IKKε/TBK1 pathway.

Genomic expression level of IRF3 and IRF7 in this study showed conflict results that IRF3 reduced and IRF7 increased relatively. According to our results, IRF3 gene expression seemed to decline in MNV-infected positive control group and infected cells treated with *Lactobacillus* group within 24 hours. This results may have relationship with TLR3, the dsRNA virus recognizing receptors, stimulating activation of IRF3. Whereas after 48 hours

incubation, IRF3 expression level increased along with *Lactobacillus* treatment dose-dependently comparing to negative control. This indicates certain factor activated by *Lactobacillus* exists, and participates in IRF3 stimulating pathways through some way unknown. Or, it is known that many viral proteins interact with components of antiviral complex and subsequently inhibit phosphorylation and activation of IRFs. There is some possibility murine norovirus may impede transcription activity of IRF3 via inactivating something participated in activating IRF3 transcription cascade pathway.

Meanwhile, IRF7 may be stimulated by TLR7 or TLR8 known to recognize ssRNA. This could be reason why IRF7 expression level increased in all treatment groups within 24 hours incubation. And IRF7 also can be Interferon stimulated gene. Because it has positive feedback relationship with type Ⅰ IFN. But There are no significant differences between MNV-infected group and *Lactobacillus* treatment MNV-infected group in IRF7 expression level (data not shown).

Interferon is a representative cytokine activated by viral infection. Type Ⅰ IFNs, including IFN-α and IFN-β are key components of the host defense against viral infections and possess powerful antiviral properties. IFN α/β, released by virus-infected cells, binds to their specific IFN receptors and leads to activation of the Jak/STAT signaling pathway. Activation of STAT1 induces an IFN-mediated antiviral response through effector pathways such as the Mx-GTPase pathway, the 20,50-oligoadenylate-synthetase-directed ribonuclease L pathway, the PKR pathway. These effector pathways individually block viral transcription, degrade viral RNA, inhibit translation,

and modify protein function to control all steps of viral replication. IFNs have been used as anti-rotavirus agents[29,30]. Similarly, Park et al. (2013) reported that *L. plantarum* DK119 exhibits antiviral effects on influenza virus infection by modulating innate immunity[31]. And lee et al. (2013) reported that *B. adolescentis* SPM0212 inhibits hepatitis B virus through an IFN-mediated antiviral response[32].

As mentioned above, probiotics have the ability to modulate the immune response and antiviral activity through activation of various cytokines. To clarify the mechanism of antiviral effect of novel probiotics, we assessed whether LR50382 inhibited MNV infection through an IFN-mediated antiviral response. According to expectations, LR50382 significantly induced Type Ⅰ IFN, IFN-α/β, comparing to the control. However, there were not significantly expression higher than only MNV-infected cells in MNV-infected cell with *Lactobacillus* treatment (data not shown). But rather only MNV-infected cells expressed IFN-α4 100 fold higher than MNV-infected cells treated *Lactobacillus*. It could be interpreted that type Ⅰ IFNs were using as releasing factor to send a signal about invasion during dying in only MNV infected-RAW264.7 cells

Furthermore, we described LR50382 increased gene expression of Mx1 (also called MxA), one of the Interferon-stimulated genes. Mx1, Myxovirus-resistance proteins 1, can directly interact with vital particles in infected cells leading to their sequestration[33]. In our data, Mx1 gene expression increased after 48 hours infection except 48h incubated MNV infected-cells with 10^8 CFU/ml LR50382 treatment.

Until now, we investigated the effects of lactobacilli on innate immune response, especially Type Ⅰ IFN. We assumed IFN-α/β induction attributed to antiviral effects of *Lactobacillus* spp. However, *Lactobacillus* spp. have been studied to inhibit MNV replication by various mechanisms. The further study is needed about the metabolites of *Lactobacillus* strains, which may affect decrease of MNV replication.

As mentioned above, there are various antiviral mechanisms of probiotics. Innate immune response maybe has partial affects to inhibit viruses. Further study is necessary because the antiviral mechanisms of probiotics still remain unclear.

Ⅴ. Conclusions

Overall, our results indicated that *Lactobacillus* spp. isolated from Korean infants efficiently inhibit replication of murine norovirus in MNV-infected RAW264.7 cells. Especially, the antiviral effect of *L.reuteri* SNUG50382 is worthy of notice. *L.reuteri* SNUG50382 was conformed having ability of acid, bile-tolerance and low antibiotics resistance. We conformed its anti-noroviral effects in two different ways, plaque assay and qRT-PCR. The antiviral effects of the novel probiotics are likely due to a modulation of the immune response through promoting Interferon regulating factor 7, which are key regulators in the IFN signaling pathway.

Nevertheless, significant reduction of MNV amounts in RAW264.7 cells treated with LR50382, there remain various methods about inhibiting replication of MNV by *Lactobacillus* spp. We should investigate other pathways such as mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in further study.

Consequently, clarifying the underlying processes and the many ways in which viruses have captured and hindered by probiotics is important with regard to our general understanding of antiviral mechanisms, and is the alternative way in which we will stand reasonably prepared for the next emerging virus disease. This study suggest *L. reuteri* SNUG50382 has the potential to be a new prevention and reliever tool against norovirus gastroenteritis because of its anti-noroviral effects. However, there are still

remain unclear parts needed further study.

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국문 초록

한국인에서 순수배양된 유산균의 항노로바이러스 효과 연구

서울대학교 보건대학원

환경보건학과 환경보건학전공

고 나 윤

지도교수 고 광 표

노로바이러스 감염병은 매년 최소 20 만명의 사망자를 발생시키는 것을 포함하여 비용적, 국민보건의 측면에서 큰 손실을 발생시키고 있다. 하지만 연구기반 확충의 제한점으로 인해 효과적인 백신을 개발하는데 어려움을 겪고 있으며, 현재까지 적당한 치료법이나 예방법이 미비한 상태이다.

인간에게 유익한 살아있는 균인 프로바이오틱스는 사람의 장내에 서식하면서 면역 조절의 역할을 하는 것으로 알려져 있다. 이전 연구들에서 프로바이오틱스가 다양한 기작을 통해 항바이러스 활성을 가진다는 보고가 있어왔다. 본 연구에서는 프로바이오틱스가 선천적 면역반응 조절기능이 항바이러스효과에 미치는 영향에 집중하였다. 이를 통해 본 연구에서 분리한 유산균이 노로바이러스 감염병을 예방 및 조절할 수 있는 프로바이오틱스 치료제가 될 수 있는지 여부를 확인하고자 한다.

본 연구에서는 한국인 장내에서 락토바실러스를 분리하였고, 분리된 균들의 항 노로바이러스 효과를 확인하였다. 모든 균들이 노로바이러스의 한 종류인 쥐노로바이러스를 저감하였으나, 특히 프로바이오틱스에 필요한 조건들을 갖추고 있는 Lactobacillus reuteri SNUG50382 (LR50382)균이 노로바이러스 감염을 유의하게 저해하는 것을 실험을 통하여 확인하였다.

노로바이러스 감염병은 증상의 발병과 변화가 빠르게 일어나는

점으로 미루어 볼 때, 노로바이러스를 조절하는 데에는 제 1 차 방어 기작인 선천적 면역반응이 중요한 역할을 할 것으로 생각되고 있다. LR50382 균의 노로바이러스 저해 기작을 확인하기 위해 본 연구에서는 인터페론 신호전달체계의 구성성분들과 인터페론으로 유발되는 항바이러스 작동 인자의 유전자 발현량을 측정하였다. LR50382 균을 감염된 세포에 처리했을 때, IFN-α 와 IFN-β 의 유전자 발현량이 컨트롤 보다 유의하게 증가하는 것을 확인하였다. 또한 인터페론 조절 인자인 IRF7 과 Mx1 의 발현량도 증가하였다. 따라서, 이 결과들은 LR50382 가 세포수준에서 노로바이러스의 증식을 효과적으로 저해한다는 것을 보여주는데, 이것은 인터페론 타입 1 의 발현을 증진시켜 면역반응을 조절하는 능력이 영향을 미치는 것으로 유추할 수 있다.

주요 단어: 프로바이오틱스; 락토바실러스; 항바이러스 효과; 선천적 면역반응; 노로바이러스; 순수분리

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