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Antioxidative and Probiotic Properties of *Lactobacillus gasseri* NLRI-312 Isolated from Korean Infant Feces

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ABSTRACT : We selected a *Lactobacillus spp.* from Korean healthy infant feces based upon their antioxidant activity. This strain was identified as *Lactobacillus gasseri* by 16S rDNA sequencing, and named *Lactobacillus gasseri* NLRI-312. In the present study, we investigate the protective effect of this strain on the H_2O_2 induced damage to cellular membrane lipid and DNA in Jurkat cells. To estimate the extent of cellular lipid peroxidation inhibition, MDA (malondialdehyde) was measured, and DNA damage was tested by the comet assay. We also examined probiotic properties including tolerance to acid and bile, antibiotic resistance. From the results obtained, the supplementation of Jurkat cells with NLRI-312 decreased in DNA damage, while no effect was shown on MDA decrease. In probiotic properties, this strain was resistance to both acid and bile, showed considerably higher survival when incubated in pH 2 or 1% bile salts (w/v). We concluded that the NLRI-312 could be used as potential probiotic bacteria, with the effect of reducing DNA damage induced by H_2O_2. (**Key Words :** *Lactobacillus gasseri*, Antioxidant, Probiotic, DNA Damage, Jurkat Cell)

INTRODUCTION

Probiotics are defined as the viable microorganism that exhibit a beneficial effect on health of the host by improving its intestinal microbial balance (Fuller, 1989). *Lactobacillus* is one of the most extensively studied probiotic. Traditionally, lactobacilli have been used for dairy products such as milk or yogurt, and are considered as GRAS (Generally Recognized as Safe) organisms that can safely be used also for a pharmaceutical.

During recent years, numerous studies have been undertaken to obtain scientific evidences for beneficial effects of *lactobacillus* as promising probiotic. Such beneficial effects are considered to include the protection from pathogens, enhancement of the immune system, antimutagenic and anticarcinogenic effects, and the reduction of serum cholesterol levels (Saavedra, 2001).

Moreover, recently, some studies described the behavior of selected probiotic strains of their antioxidant activity (Kullisaar et al., 2002, Annuk et al., 2003). It has been suggested that *Lactobacillus* may improve the antioxidant defence system of the host.

However, there are few studies on the protective effect of *Lactobacillus* against oxidative damage in cell culture. Evaluation of antioxidant activity by the method based on cell cultures seems to be more appropriate than by the method of lipid peroxidation (Bei et al., 2004).

To provide health benefits, probiotic must be capable of surviving and colonizing the intestinal tract (Lee and Salminen, 1995). In order to survive in and colonize the gastrointestinal tract, probiotic should express high tolerance to acid and bile (Kirjavainen et al., 1998).

From this point of view, we screened *Lactobacillus spp*. from Korean healthy infant feces for their ability to survive in the acid and bile conditions. The promising strains were further screened for their antioxidant activity by the inhibition of lipid peroxidation. Finally, a *Lactobacillus spp*. with high antioxidant activity was selected. This strain was identified as *Lactobacillus gasseri* by 16S rDNA sequencing and named *Lactobacillus gasseri* NLRI 312.

In the present study, we investigated the protective effect of the selected *Lactobacillus gasseri* NLRI 312 against oxidative damage to cellular membrane lipid and DNA in Jurkat cell line. We also evaluated the probiotic properties including acid tolerance, bile resistance, and antibiotic resistance.

MATERIALS AND METHODS

Isolation of *Lactobacillus gasseri* NLRI 312

The *Lactobacillus gasseri* NLRI 312 had been isolated

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from feces of new-born infants at a obstetrics and gynecology of the city of Suwon, Korea. Tenfold serial dilutions of the fecal samples were prepared with physiological saline, and 0.1 ml of each dilution was plated onto MRS agar (Difco). The plate incubated at 37°C for 2 days in an anaerobic chamber (BBL anaerobic system). Representative colonies were selected on the basis of colony morphology, microscopy, gram-positive, and catalase-negative. Isolates were screened for their antioxidant activity by inhibition of linoleic acid peroxidation system. *L. acidophilus* KCTC 3111, *L. acidophilus* KCTC 3151 and *L. brevis* KCTC 3498 were used as reference strains for probiotic properties. Antioxidant properties of these strains were characterized in our previous study (Kim et al., 2006). Lactobacilli strains were kept in MRS broth plus glycerol (70:30) at -80°C and were sub-cultured three times in MRS broth for activation prior to experimental use.

Total DNA preparation, 16S rDNA, and PCR sequencing

The DNA isolated by using a bead beater apparatus. The samples were placed in 300 μl of Tris-EDTA buffer, beaten with 0.25 g of 1 mm glass beads for 60 s at 5,000 rpm in bead-beater (Mini-Bead-Beater, Biospec Products), and chilled on ice immediately thereafter. The DNA recovered by precipitation of the final aqueous phases with ammonium acetate and ethanol, centrifuged at 14,000×g. 25°C, for 10 min, then suspended in Tris-EDTA (pH 7.4) buffer and stored at -20°C.

16S rDNA gene form the isolate was isolated PCR amplification from boiled cells of an overnight culture by using the forward primer 5′-GAGTTTGATCCTGG CTCAG-3′(*E. coli* 9-27) and a reverse primer 5′-AGAAAG GAGGTGATCCAGCC-3′ (*E. coli* 1542-1525). Conditions for PCR were 95°C 1 min; 60°C 1 min; 72°C 1 min, 35 cycles. An aliquot of PCR products was analyzed by gel electrophoresis to confirm the presence of the correct size product. Transformation was done by using compentent cells (*E. coli* DH5 F′). The transformed cells were plated on Luria-Bertani agar plates supplemented with ampicillin and incubated overnight at 37°C. Colonies were placed in 40 μl of 10 mM Tris. The sizes of the inserts were determined by PCR with flanking vector primers followed by electrophoresis in a agarose gel.

Purified DNA from PCR was sequenced by using a genetic analyzer 355 (Perkin-Elmer, USA) according to the manufacturer's instructions (Weisburg et al., 1991). Based on the search results, similar 16S rDNA gene sequences were aligned with isolate using CLUSTAL W computer program (Thompson, Germany) and PHYLIP program (Felsenstein, USA) (Tompson et al., 1994).

Preparation of cell lysate of *Lactobacillus gasseri* NLRI 312

The cells were harvested by centrifugation at 4°C for 30 min (4,000×g) and the pellet was washed twice with 20 mM sodium phosphate buffer (SPB, pH 7.4) and then resuspened in SPB. Washed cell suspension was disrupted at 4°C by ultrasonic disruption. Cell debris were removed by centrifugation (7,000×g for 10 min at 4°C), and filtration (0.45 μm, Millipore). Protein concentration was measured by Bradford method (Bio-Rad Laboratories) after adjust to 1 mg/ml.

Antioxidant properties

Antioxidant activity by inhibition of linoleic acid peroxidation : The antioxidant activity was determined according to the TBA (thiobarbituric acid) method (Lin and Chang, 2000) and described in a previous study (Kim et al., 2005). Briefly, reaction mixtures contained 0.6 ml of 20 mM sodium phosphate buffer (pH 7.2), 1 ml linoleic acid emulsion (0.1 ml linoleic acid; 99%, Sigma, 0.2 ml Tween 20 and 19.7 ml deionized water) and 0.2 ml of sample. Lipid peroxidation was started by the addition of $FeSO_4$ (0.2 ml, 0.01%) and H_2O_2 (0.2 ml, 0.56 mM). The reaction mixture was incubated in water bath for 6 h at 37°C. Trichloroacetic acid (TCA; 0.2 ml, 4%), TBA (2 ml, 0.8%) and butylated hydroxytoluene (BHT; 0.2 ml, 0.4%) were then added, and the mixture was boiled at 100℃ for 20 min. The antioxidant activity was evaluated from the extracted peroxide by measuring the absorbance at 532 nm using Jasco V530 spectrophotometer (Japan), and expressed as the percentage of inhibition of linoleic acid peroxidation with a control containing no sample.

Antioxidant activity in Jurkat cell :

i) Cell culture and H_2O_2 treatment

The jurkat cell line (ATCC TIB-152) was used in this study. This cell line has been widely used as target cells to investigating cell response to oxidative stress (Duthie et al., 1996, Riso et al., 1999).

Cell was cultured in RPMI 1640 (BioWhittaker, USA) containing 2 mM of L-glutamine, 1×10^5 U/L of penicillin, 100 mg/L of streptomycin, and 100 ml/L of fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. Medium was changed every 48 h, and the cell number was adjusted to 5×10^5cell/ml after hemocytometer counts.

The cell lysate of NLRI 312 was dissolved in phosphate buffered saline (PBS, BioWhittaker) and added to cell culture and maintained for 12 h at 37°C. After the supplement, cell suspension was washed twice with PBS and put in fresh medium before the treatment with H_2O_2. The oxidative treatment was performed by adding 100 μM/ml of H_2O_2 for 30 min at 37°C. Then cells were centrifuged and wash with PBS, and were sonicated and

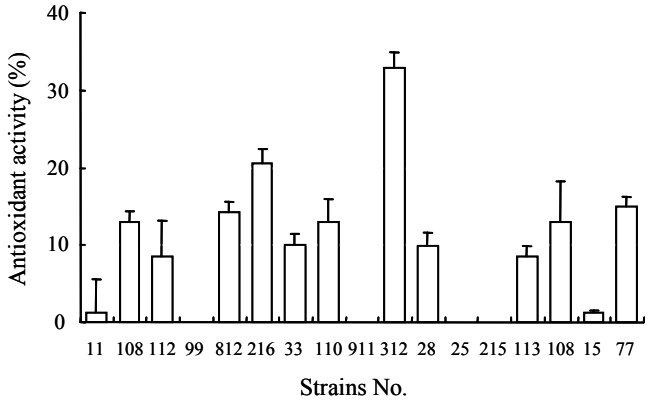

Figure 1. Antioxidant activity of 17 selected *Lactobacillus* strains.

centrifuged at 3,300×g for 10 min at 4°C. The supernatants were used in the assay. Protein concentration was determined by the Bradford method (Bio-rad).

ii) Measurement of MDA (malondialdehyde)

MDA, a terminal product of lipid peroxidation was measured to estimate the extent of cellular lipid peroxidation in Jurkat cell. Measurements of MDA were performed following the instruction of reagent kits (Calbiochem®). A calibration curve of 1,1,3,3,-tetramethoxypropane (0-20 μM) was used as a standard and the results were expressed as μM MDA/mg protein.

iii) Determination of DNA damage by comet assay (single cell gel electrophoresis)

The comet assay was used to evaluate DNA damage in control and supplemented Jurkat cells that were or were not subjected to the H_2O_2 treatment. For this, cells were embedded in agarose on frosted slides, lysed, and then subjected to electrophoresis under alkaline conditions (Singh et al., 1998). After neutralization, cells were stained with ethidium bromide, and microscopical analysis revealed the images of damaged DNA (comets). The production and extend of DNA migration were determined for 50 DNA spots per slide. Cell images were electro-analyzed for fluorescence intensity with comet analysis program. The DNA damage was expressed as percentage of DNA in tail.

Probiotic properties

Acid tolerance : To determine the transit tolerance to low pH, the method of Pennacchia et al. (2004) was used with slight modifications. Strains were grown in MRS broth at 37°C for 24 h. A 0.5 ml aliquot of the bacterial culture was inoculated in 10 ml of phosphate buffered saline adjusted to pH 2 with 4 N HCl. Phosphate buffer was prepared by dissolving NaCl (9 g/L), $Na_2HPO_4 \cdot 2H_2O$ (9 g/L), and KH_2PO_4 (1.5 g/L) in distilled water. The initial bacterial concentration was $10^{6\sim8}$ cfu/ml. Cultures were incubated at 37°C. After 0, 0.5, 1, 2 and 4 h incubation, cells were serially diluted tenfold in 0.1 M sodium phosphate buffer (pH 7.2), and the viable cells were enumerated on MRS agar plate at 37°C for 2 days.

Bile resistance : The resistance to bile was examined according to method of Gilliland and Walker (1990). Each strain was inoculated in MRS broth with 0.5 or 1% (w/v) bile salt (Difco). Cultures were incubated at 37°C for 24 h, and the numbers of viable cell were determined and compared to a control (without bile salts) on MRS agar plates.

Antibiotic resistance : Antibiotic resistance of strains was tested by the agar disc diffusion method. Inhibition zone diameters were measured after incubation at 37°C for 24 h, and results were expressed in terms of resistance strain (R), intermediate sensitive strain (I) or sensitive strain (S). Antibiotic discs (senti-disc™, BBL, USA) were Kanamycin (30 μg), erythromycin (15 μg), penicillin G (10 U), tetracycline (30 μg), chloramphenicol (30 μg), vancomycin (30 μg), and streptomycin (10 μg).

Statistical analysis

The results were expressed as means±SD. Statistical analysis was performed by one-way analysis of variation (ANOVA), and significant differences were detected ($p<0.05$) by Duncan's multiple range tests or student's t-test using a PC statistical package (SAS, release 8.01, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Isolation and identification

Before screening of *Lactobacillus spp*. with antioxidant activity from infant fecal samples, we determined of strains resistance to 0.3% bile salts. In the present study, similar tolerance to 0.3% bile salts were observed among the strains tested. A surviving incubation at low pH (pH 2.5) was set for further screening. Among 32 strains tested for pH resistance, 17 strains exhibited resistance to acid, which showed a survival percentage over the 80% when incubated at pH 2.5 for 2 h. And then further isolation of the strains was based upon the antioxidant activity by linoleic acid peroxidation system.

As shown in Figure 1, the antioxidant activity also observed to be variable among the 17 selected strains. Many of the strains showed weak or no antioxidant activity. NLRI-312 showed the highest antioxidant activity. This strain was identified as *Lactobacillus gasseri* by 16S rDNA sequencing, and named *Lactobacillus gasseri* NLRI -312. In subsequent experiments, this bacterial isolate was extensively studied for protective effect of oxidative damage to lipid and DNA in Jurkat cells. Also we evaluated probiotic properties.

Antioxidant activity

Antioxidant activity in linoleic acid peroxidation :

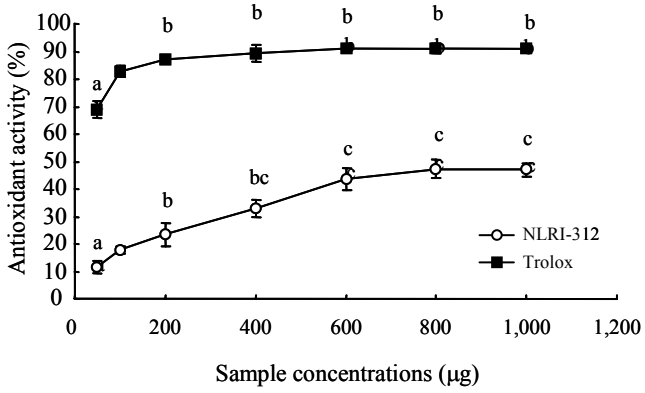

Figure 2. Antioxidant activities at different concentrations of NLRI-312 and Trolox as a standard antioxidant. [a-c] Values in the same column with different superscripts letters are significantly different ($p<0.05$).

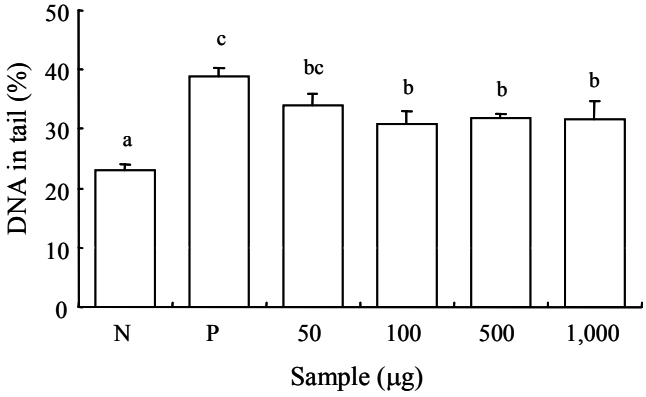

Figure 3. DNA damage evaluated by the comet assay and expressed as DNA in tail (%).N: negative control (no H_2O_2), P: positive control: (100 μM/ml H_2O_2). [a-c] Values in the same column with different superscripts letters are significantly different ($p<0.05$).

Figure 2 shows the antioxidant activity of cell lysate of NLRI 312 and trolox at different concentrations (50-1,000 μg protein). The antioxidant activity was found to increase with the increasing of NLRI-312 concentration, and reached to maximum antioxidant activity at the concentration of 600 μg NLRI-312. The antioxidant activity was not significantly increased with more concentration. The percentage of antioxidant activity at the concentration of 600 μg was found as 43.7%, while the same concentration, Trolox, used as the standard, exhibited 91.2%.

Antioxidant activity in Jurkat cell line :

i) Measurement of MDA

To investigate the effect of NLRI-312 on preventing cellular lipid peroxidation induced by H_2O_2, MDA production as a marker of cellular membrane lipid oxidative damage in Jurkat cells was measured. Supplementation with NLRI-312 had no effect on the MDA production (data not shown). Also, positive control cells treated with H_2O_2 did not increased MDA production.

The possible explanations could be given to this observation is that, the oxidation of Jurkat cells with 100 μM/ml H_2O_2 seems to be not appropriate to induce cell membrane lipid damage and to produce detectable levels of MDA.

H_2O_2 has been extensively used to induce oxidative damage *in vitro* models (Xiao et al., 2000; Tanaka et al., 2001). It is known that H_2O_2 itself is not reactive but can penetrates into cells and induce hydroxyl radicals that attack components including lipids, proteins and DNA to cause a wide variety of oxidative insults (Halliwell and Gutteridge, 1990). But, in our study H_2O_2 did not increased MDA production. Also Erba et al. (1999) reported that MDA levels increase with the concentration of Fe^{2+}, whereas no MDA increase was found after H_2O_2 treatment. Therefore, H_2O_2 seemed not reliable oxidant species to study lipid peroxidation in cell culture.

Determination of DNA damage : The protective effect of NLRI-312 on DNA damage induced H_2O_2 was investigated in Jurkat cells by using comet assay, which is a useful and sensitive procedure for detecting DNA single strand breaks in any population of cells (Erba et al., 1999; Riso et al., 2002).

As shown in Figure 3, treatment with 100 μM/ml H_2O_2 caused significant increase in DNA damage with respect to negative control cells. On the contrary, cells previously supplemented with NLRI-312 were protected from oxidative damage. The reduction of DNA damage with respect to positive control was about 50% after 50 μg NLRI-312 supplementation. It should be noted that the protective ability of NLRI-312 seems not strictly dose dependent, at least at the concentrations used in the present study. This finding is interesting because it suggests that NLRI-312 can prevent DNA damage independently from lipid peroxidation. Oxidative treatment can also induce DNA damage. It has been suggested that substantial oxidative DNA damage *in vivo* contributes to the etiological cancer. Consequently, the quantification of DNA damage is a useful biomarker of the oxidative stress and the antioxidant defense system of the cell (Riso et al., 1999).

The results we obtained support the protective effect of NLRI-312 against oxidative damage in Jurkat cells.

Probiotic properties

Acid tolerance : The growth of the NLRI -312 and three reference strains in phosphate buffer at pH values of 2.0 and 3.0 was investigated. The *lactobacillus* strains showed different resistance to low pH. As shown in Figure 4, NLRI-312 strain was the most acid resistant, and survived at pH values as low as 2.0 for at least 2 h. While *L. acidophilus* KCTC 3151 and *L. brevis* KCTC 3498 strains showed to have very poor survival when exposed to low pH solutions.

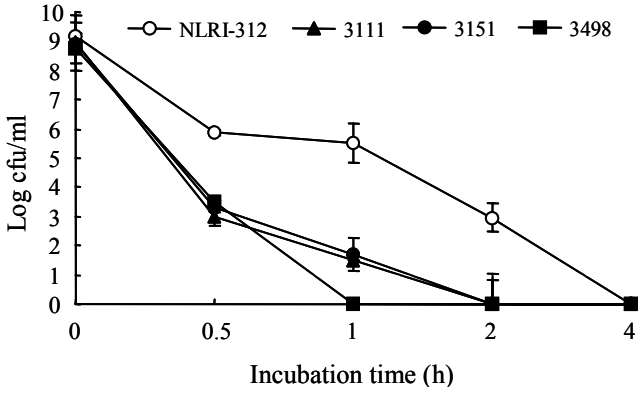

Figure 4. Survival of the *Lactobacillus* strains in pH 2. * Indicate a significant difference from the reference strains ($p<0.05$). NLRI-312: *L. gasseri* NLRI-312, 3111: *L. acidophilus* KCTC 3111, 3151: *L. acidophilus* KCTC 3151, 3498: *L. brevis* KCTC 3498.

Especially, *L. brevis* KCTC 3498 was sensitive to low pH since survival was observed at pH 3 only (data not shown).

The ability of probiotic bacteria to survive the passage through the stomach was reported to be variable and strain dependent (Clark et al., 1993; Charteris et al., 1998). Conway et al. (1987) suggested that these differences in the acid and bile tolerance of strains from species might be due to difference in the cell wall structure.

The gastric pH in healthy humans is about 2-2.5. Which causes the destruction of most microorganisms ingested (Lankaputhra, 1995). In this sense, resistance to human gastric transit is an important selection criterion for probiotic bacteria (Charteris et al., 1998; Kimoto et al., 2000). Gilliland et al. (1984) stated that *lactobacillus* strains with high acid and bile resistance showed better growth and colonization character in the small intestine than non-resistant strains.

Acid tolerance of probiotics is important not only for resistance to acid, but is also prerequisite for their use as dietary adjuncts and enables strains to survive for longer periods of time in high acid carrier foods such as yogurt, without large reduction in viable cell numbers.

Resistance to bile : The resistance of the strains to high bile conditions was determined. Table 1 showed that most strain grew in the presence of bile at 1.0%, during incubation for 24 h. The survival of *Lactobacillus* strains at bile concentrations of 0.5 and 1% were very similar (Data not known). Our results indicate that NLRI-312 shows more resistant than all the other strains. In the presence of 1% bile salts, NLRI-312 was increased by 2.11 log cfu/ml for 24 h incubation. On the other hand, *L. acidophilus* KCTC 3111 showed a high degree of sensitivity, and was increased by 1.12 log cfu/ml with bile salts, while by 2.94 log cfu/ml without bile salts.

Resistance to bile salts is a prerequisite for colonization and metabolic activity of probiotic bacteria in the small intestine of the host (Havenaar et al., 1992). The relevant physiological concentrations of human bile range from 0.3% (Dunne et al., 2001) to 0.5% (Zavaglia et al., 1998). Bile resistance of *Lactobacillus* strains is typically determined at bile concentrations between 0.1 and 1% w/v in the growth medium (Jacobsen et al., 1999; Hyronimus et al., 2000; Xanthopoulos et al., 2000). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes (Gilliland et al., 1984). In this sense, it is generally considered necessary to evaluate the ability of potential probiotic bacteria to resist the effects

Table 1. Effect of bile salts concentration on growth of the *Lactobacillus* strains

Strains	Viable counts (log10 cfu/ml)					
	0% bile salts			1% bile salts		
	0 h	24 h	Increase	0 h	24 h	Increase
L. gasseri NLRI-312	6.97±0.05	9.61±0.14	2.64	6.85±0.14	8.96±0.02	2.11*
L. acidophilus KCTC 3111	7.36±0.21	10.3±0.10	2.94	7.46±0.31	8.58±0.36	1.12
L. acidophilus KCTC 3151	7.25±0.52	10.15±0.20	2.9	7.32±0.15	8.9±0.25	1.58
L. brevis KCTC 3498	7.04±0.11	9.6±0.38	2.56	7.00±0.44	8.59±0.80	1.59

Increase = $\log_{10}$(final population)-$\log_{10}$(initial population).

* Indicate a significant difference from the reference strains ($p<0.05$).

Table 2. Antibiotic resistance of the *Lactobacillus* strains

Antibiotic	Strains			
	L. gasseri NLRI-312	*L. acidophilus* KCTC 3111	*L. acidophilus* KCTC 3151	*L. brevis* KCTC 3498
Kanamycin (30 μg)	R	I	R	R
Erythromycin (15 μg)	S	S	S	S
Penicillin G (10 U)	S	S	S	I
Tetracycline (30 μg)	S	S	S	I
Chloramphenicol (30 μg)	I	S	S	S
Vancomycin (30 μg)	I	R	R	R
Streptomycin (10 μg)	I	I	I	I

R: resistant strain, I: intermediate sensitive strain, S: sensitive strain.

of bile salts (Collins et al., 1998).

The NLRI-312 not only survived at the low pH condition but also grew at bile salt concentrations up to 1% (w/v).

Antibiotic resistance : The antibiotic resistance of the strains was shown in Table 2. The NLRI-312 strain was resistant to kanamycin and showed an intermediated susceptibility to chloramphenicol, vancomycin and streptomycin. Reference strains showed resistant to vancomycin. All strains were sensitive to erythromycin. Xanthopoulos et al. (2000) and Arici et al. (2004) showed lactobacilli strains of infant feces were sensitive against erythromycin, penicillin and tetracycline. These findings were similar to our results.

CONCLUSION

We investigate the protective effect of *Lactobacillus gasseri* NLRI-312 on the H_2O_2 induced damage to cellular membrane lipid and DNA in Jurkat cells, which strain was selected from Korean healthy infant feces based upon their antioxidant activity. To estimate the extent of cellular lipid peroxidation inhibition, MDA (malondialdehyde) was measured, and DNA damage was tested by the comet assay. We also examined probiotic properties including tolerance to acid and bile, antibiotic resistance. We concluded that the *Lactobacillus gasseri* NLRI-312 strains not only survival the low pH but also normal growth at bile concentration up to 1%. And the NLRI-312 showed protective effect on DNA oxidative damage at low concentration as 50 μg of protein.

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