Food Sci. Biotechnol. Vol. 16, No. 5, pp. 843 ~ 847 (2007)

Food Science and Biotechnology

Potential Probiotic Properties of *Lactococcus lactis* NK34 Isolated from *Jeotgal*

Na-Kyoung Lee, Ji Eun Noh, Gui-Hun Choi, Eunju Park[1], Hyo-Ihl Chang[2], Cheol-Won Yun[2], Seung-Wook Kim[3], Chang-Won Kang, Yoh-Chang Yoon, and Hyun-Dong Paik*

Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea
[1]Department of Food and Nutrition, Kyungnam University, Masan, Gyeongnam 631-701, Korea
[2]School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea
[3]Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Korea

Abstract Strain NK34 was characterized for probiotic use. Strain NK34 was named *Lactococcus lactis* NK34 based on API 50 CHL kit results and 16S rDNA sequencing. *L. lactis* NK34 was highly resistant to artificial gastric juice (pH 2.5) and artificial bile acid. Based on results from the API ZYM kit, 4 enzymes were produced. *L. lactis* NK34 was resistant to all antibiotics tested except for 10 μg/mL roxithromycin and 10 μg/mL erythromycin. The cholesterol-lowering effect of *L. lactis* NK34 was about 46.9%. Concentrations of interleukin (IL)-1α in the 20× concentrated supernatant of *L. lactis* NK34 was about 361 pg/mL. *L. lactis* NK34 was also found to inhibit the growth of colon cancer cells due to MNNG-induced DNA damage. These results demonstrate the potential of *L. lactis* NK34 as a health-promoting probiotic.

Keywords: *Lactococcus lactis, jeotgal*, probiotics, cholesterol-lowering activity, immunolstimulating activity, DNA damage

Introduction

Probiotics are defined as live microbial feed supplements which have beneficial effects in the host animal by improving its intestinal microbial balance (1-6). Also, probiotics was used for human as dairy products and health food. Probiotic characteristics that have been studied include survival in gastric conditions, colonization of the intestine, reduction of lactose intolerance, prevention of antibiotic-induced diarrhea, prevention of colon cancer, and stimulation of the immune system (7-12).

Lactic acid bacteria (LAB) are GRAS (generally recognized as safe) organisms, and LAB probiotics typically use *Lactococcus acidophilus*, *Lactococcus casei*, *Lactococcus reuteri*, and so on (13). *Lactococcus* spp. are widely used as starter bacteria in manufacturing cheese and other fermented dairy products. Establishing the effective probiotic properties of *Lactococcus* spp. could lead to the development of new probiotic foods. For example, *Lactococcus lactis* L1A is used as commercial probiotic products in yogurt in Sweden (13), and *L. lactis* MG 1363 was studied regarding the delivery of vaccinal epitopes in the gastrointestinal tract (14).

Strain NK34 isolated from *jeotgal*, a Korean fermented fish food, has been reported as a bacteriocin producer (15, 16). To study the potential probiotic properties of *L. lactis* NK34, we investigated its resistance to artificial gastric and bile acids, enzyme productivity, antibiotic resistance, cholesterol-lowering effects and immunostimulatory activity.

*Corresponding author: Tel: 82-2-2049-6011; Fax: 82-2-455-3082
E-mail: hdpaik@konkuk.ac.kr
Received July 21, 2007; accepted August 30, 2007

Materials and Methods

Bacterial strain and culture media *L. lactis* NK34 was isolated previously from *jeotgal*, and this strain was used in this study (15). This strain was grown in lactobacilli MRS broth (Difco Lab., Detroit, MI, USA) at 37°C, and stored as stock solutions in 20%(v/v) glycerol at -70°C.

Identification of strain NK34 Cell morphology, Gram-staining, API 50 CHL medium and 16S rDNA sequences were analyzed for the identification of strain NK34. API 50 CHL medium (BioMerieux, Lyon, France) was used to study the carbohydrate usage by the strain. 16S rDNA sequence data was obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea).

Tolerance to artificial gastric juice and artificial bile acid Analysis of artificial digestive fluid tolerance followed the method of Kobayashi *et al.* (17, 18). Initially, cells were harvested by centrifugation at 4,000×g at 4°C for 10 min. *L. lactis* NK34 was then suspended in MRS broth containing 1%(w/v) pepsin, adjusted to pH 4.0 and 2.5 with 0.1 M HCl and cultured for 2 hr at 37°C. Artificial bile acid tolerance was determined by cultivating cells treated with artificial gastric juice (pH 2.5). The cells were incubated for 24 hr at 37°C in artificial bile acid consisting of MRS broth containing 0.1%(w/v) oxgall (Difco Lab.). Viable cells were measured by incubating aliquots on MRS agar plates for 24 hr at 37°C.

Enzyme activity The API ZYM kit (BioMerieux) was used to study enzyme activity. *L. lactis* NK34 was grown overnight at 37°C on MRS agar. Sediment from centrifuged culture broth was used to prepare the suspension at 10^5 CFU/mL. After inoculation, cultures were incubated for 4 hr at 37°C. Placing a surface-active agent (ZYM A

reagent) in the cupules facilitated solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values from 0-5 were assigned corresponding to the colors developed. The approximate nmol of free hydrolyzed substrate was determined based on the color strength: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 nmol or higher.

Antibiotic sensitivity Antibiotic sensitivity was determined by the paper disc method. Soft agar (0.75%, w/v), containing 10^7 *L. lactis* NK34 cells was overlaid on agar plates. After the medium had solidified, a sterile paper disk previously treated with antibiotics diluents was laid on the agar, and the plate was incubated at 37°C for 24 hr. The zone of inhibition was measured from the edge of the disk.

Cholesterol-lowering effect Samples (1 mL) were centrifuged (10 min, 4,000×g) and supernatants were used directly for analyses. The concentrations of total cholesterol in supernatants were determined enzymatically with the BCS total cholesterol kit (Bio Clinical System Co., Seoul, Korea).

Production of interleukin (IL)-1α *L. lactis* NK34 supernatant was concentrated by ethanol precipitation. IL-1α in 20×concentrated supernatants was measured by quantitative enzyme-linked immunosorbent assay (ELISA) with commercially available kits (Koma Biotech Inc., Seoul, Korea). The color reaction was measured at 450 nm on an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of DNA damage Phagocytic cells from healthy volunteers (age 20-25, non-smoking) were used to assess protection from DNA damage. First, lyophilized *L. lactis* NK34 was suspended in Hank's balanced salt solution (HBSS) at a concentration of 0, 10, 25, 50, 100 mg/mL. Each suspension was then preincubated with MNNG (Fluka Co., Buchs SG, Switzerland) at a final concentration of 50 μg/mL for 30 min in a shaking incubator (37°C, 150 rpm). For the negative control, HBSS without bacterial suspension was treated with phosphate conditions. Bacteria were then removed by centrifugation at 11,000×g for 10 min and aliquots (10 μL) of the supernatants (preincubation mixture), containing unidentified metabolites of *L. lactis* NK34 and MNNG (0.5 μg/mL), were used for cell treatments. Phagocytic cells were grown in preincubation mixture for 30 min at 37°C in a shaking incubator. Cells were checked for viability by trypan blue exclusion. Cell viabilities measured immediately after each treatment always exceeded 95%.

The alkaline comet assay was conducted as described by Singh *et al.* (19) with slight modification. Briefly, a cell suspension mixed with low melting agarose gel (LMA) was added to slides precoated with 1.0% normal melting agarose (NMA). After the agarose had solidified, the slides were covered with another 75 μL of 0.5% LMA and then 0.01 M Tris, and 1% sodium laurylsarcosine for 1 hr at 4°C. The slides were then placed into an electrophoresis tank containing 0.3 M NaOH and 0.01 M Na_2EDTA (pH 13.0) for 40 min, to allow the DNA to unwind. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C, and the slides were then treated with ethanol for another 5 min before being stained with 50 μL of ethidium bromide (20 μg/mL). Fifty cells from each of 2 replicate slides were processed for each treatment group. The cells were scored for the parameter 'percentage fluorescence in tail', using image analysis (Komet 5.0; Kinetic Imaging, Liverpool, UK) and fluorescence microscopy (Leica DMLB, Benshim, Germany).

Data were analyzed using the SPSS package for windows (Version 10). Values were expressed as mean ± standard error (SE). The mean values of the data from the control group and the *L. lactis* NK34 supplement group were compared using Student's *t*-test and differences were considered significant at $p<0.05$.

Results and Discussion

Microbiological identification of strain NK34 Strain NK34 was shown to have coccus morphology and to be Gram-positive. Strain NK34 was also shown to have 99.8 % similarity to *L. lactis* in terms of its biochemical carbohydrates fermentation pattern using the API 50 CHL kit (data not shown). In addition, a 1,462 bp 16S rDNA

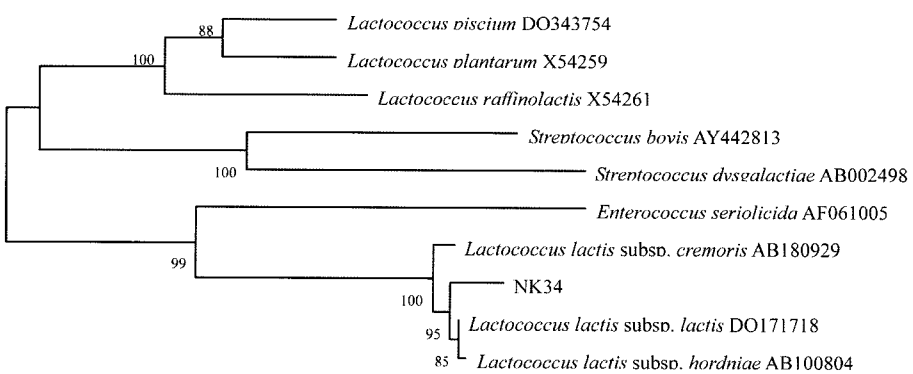

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the position of strain NK24 and representatives of some related taxa. The scale 0.1 substitution per nucleotide position.

sequence from strain NK34 had 99% similarity to that of *L. lactis*. As a result, strain NK34 was identified as *L. lactis* based on its carbon source usage and its phylogenetic tree (Fig. 1) based on 16S rDNA sequence, and was named as *L. lactis* NK34.

Tolerance to artificial gastric juice and artificial bile acid Probiotic bacteria must survive the harsh environments of the stomach (low pH) and intestinal tract, which contains bile acid. At pH 2.5, the viability of *L. lactis* NK34 decreased as the value of 1 log CFU/mL during 2 hr of incubation (Fig. 2a). Also, *L. lactis* NK34 showed almost a 100% survival rate after 24 hr incubation in artificial bile acid (Fig. 2b). *L. acidophilus, L. bulgaricus, L. rhamnosus, L. reuteri* HY701, and *Streptococcus thermophilus* are able to grow well at low pH (11, 18, 19). Lactic acid starter bacteria, *L. lactis* 13-3, 15-1, 15-4, C12, SL3, SD5, and A4 were reported to have high viability over 6.0 log of viable cells in gastric solution of pH 2 (10). *L. lactis* strains except for 13-3 have survival rates below 60% in the presence of 0.3% bile. However, *L. lactis* NK34 showed a high survival rate of over 6 log of viable cells in artificial gastric acid (pH 2.5) and artificial bile acid (0.1% oxgall) without loss of activity (Fig. 2).

Enzyme activity *L. lactis* NK34 produced enzymes such as crystine arylamidase, α-chymotrypsin, β-galactosidase, and β-glucosidase (Table 1). The enzyme production of *L. lactis* NK34 was one of the most important criteria in its selection, because carcinogenic enzymes such as β-glucuronidase can be produced by microorganisms (23). When carcinogenic substances such as a benzo(a)pyrene enter the human body, their poisonous effects are counteracted due to conjugation with glucuronic acid in the liver. If this conjugated product is excreted with bile acid in the intestine, cleavage by β-glucuronidase can liberate these substances to become toxic once again. *L. lactis* NK34 did not produce the carcinogenic enzyme, β-glucuronidase, whereas enzymes such as crystine arylamidase, α-chymotrypsin, β-galactosidase, and α-glucosidase were produced.

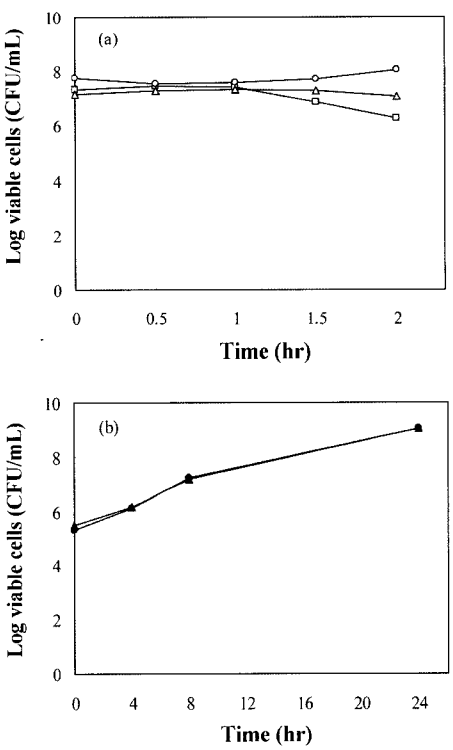

Fig. 2. Survival of *L. lactis* NK34 in (a) artificial gastric juice, (b) treated with artificial bile acid after artificial gastric juice treatment (pH 2.5) at 37°C for 2 hr. - ○ - no artificial gastric juice treatment (control); - △ - treated with artificial gastric juice (pH 4.0); - □ - treated with artificial gastric juice (pH 2.5); - ● - no bile acid added after treatment with artificial gastric juice (control); - ▲ - treated with artificial gastric juice (pH 2.5) and bile acid.

Antibiotic sensitivity Antibiotics is important to the health care industry for fighting bacterial infections. But bacteria are capable of developing resistance to antibiotics. *L. lactis* NK34 showed a broad resistance to tested antimicrobials. *L. lactis* NK34 exhibited tolerance to most antibiotics tested except for ≥10 μg/mL roxithromycin and 10 μg/mL erythromycin (Table 2). The strains did not contain any of the transferable, acquired resistance factors known to occur among lactic acid bacteria, including resistance toward chloramphenicol, erythromycin, and tetracycline (24).

Cholesterol-lowering activity During 12 hr of incubation, *L. lactis* NK34 cells increased while the cholesterol concentration decreased by over 46.9% (data not shown). The concentration of cholesterol remained unchanged during incubation at 37°C for 12 hr in MRS medium without bacteria. *L. lactis* NK34 was shown to cause the

Table 1. Enzyme activities of *L. lactis* NK34 as determined by the API ZYM kit

Enzymes	*L. lactis* NK34[1)]
Control	0
Alkaline phosphatase	0
Esterase(C4)	0
Esterase lipase(C8)	0
Lipase(C14)	0
Leucine arylamidase	0
Valine arylamidase	0
Crystine arylamidase	2
Trypsin	0
α-Chymotrypsin	2
Acid phosphatase	0
Naphthol-AS-BI phosphohydrolase	0
α-Galactosidase	0
β-Galactosidase	3
β-Glucuronidase	0
α-Glucosidase	2
β-Glucosidase	0
N-Acetyl-β-glucosaminidase	0
α-Mannosidase	0
α-Fucosidase	0

[1)]0, 1, 2, 3, 4, and 5 are 0, 5, 10, 20, 30, and >40 nmole, respectively.

Table 2. Antibiotic resistance of *L. lactis* NK34

Antibiotics	(μg/mL)	*L. lactis* NK34[1)]
Nisin	0	+
	25	+
	50	+
	100	+
Streptomycin	0	+
	5	+
	10	+
	20	+
Neomycin	0	+
	5	+
	10	+
	20	+
Roxithromycin	0	+
	5	+
	10	-
	20	-
Chloramphenicol	0	+
	5	+
	10	+
	20	+
Gentamycin	0	+
	5	+
	10	+
	20	+
Rifampicin	0	+
	5	+
	10	+
	20	+
Erythromycin	0	+
	5	+
	10	-
	20	-

[1)]+, growth; -, no growth.

reduction of cholesterol in the medium. This may be due to the physiological actions of the end products of short-chain fatty acid fermentation (especially propionate), cholesterol assimilation by the bacteria, cholesterol binding to the bacterial cell wall, the influence of bile acids to reduce cholesterol, disintegration in cell by cholesterol oxidase, inhibition of cholesterol absorption in the intestine and enzymatic deconjugation of bile acids (25).

Immunostimulatory activity Interest in the immunostimulatory effects of probiotic bacteria has increased (26). Immunostimulatory effects were determined by measuring antimicrobials and anticarcinogenic effects, and the production of nitrite and cytokines (Il-1α, Il-1β, and etc.). *L. lactis* NK34 was reported to have broad antimicrobial effects (15) and production of Il-1α and anticarcinogen were also studied. By twenty times of concentrated supernatant of *L. lactis* NK34 was shown to contain 361 pg/mL of IL-1α. Immunostimulatory activity was studied through IL-1α and DNA damage.

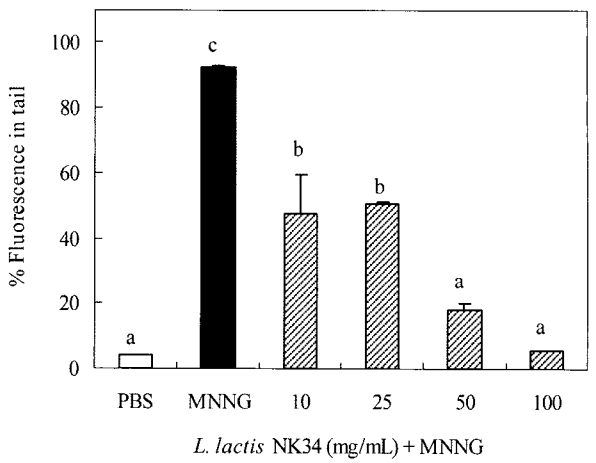

Fig. 3. Antigenotoxic effects of *L. lactis* NK34 regarding DNA damage in lymphocytes by the comet assay. Different letters above the bars indicate significant differences of α=0.05 by Duncan's multiple range test.

Figure 3 indicates the antigenotoxic effect of *L. lactis* NK34 with regard to MNNG treatment of phagocytic cells by the comet assay. DNA damage induced by MNNG was significantly inhibited by preincubating MNNG together with *L. lactis* NK34 in a dose dependent manner. *L. lactis* NK34 treatment significantly decreased comet tail intensity by 17.8 and 5.7% relative to the positive control at 50 and 100 mg/mL, respectively. Notably, the extent of comet formation in cells treated with 50 mg/mL of *L. lactis* NK34 was lower than that seen in the PBS-treated negative control. When MNNG and *L. lactis* NK34 were administered directly to the cells without any preincubation, significant effects of *L. lactis* NK34 were detectable at concentrations of 50 and 100 mg/mL. These results showed that *L. lactis* NK34 has the potential to inhibit DNA damage induced by the carcinogen MNNG, i.e., the initial stage of carcinogenesis. Also, these results provide new insights into the mechanism of the anti-cancer properties of *L. lactis* NK34. Our studies have an important bearing in evaluating the nutritional and therapeutic values of *L. lactis* NK34.

We conclude that *L. lactis* NK34 has many interesting probiotic properties, such as high survival in artificial gastric and bile acid, the production of beneficial enzymes, tolerance to antibiotics, cholesterol-lowering effects, the production of IL-1α, and inhibition of the formation of preneoplastic lesions.

Acknowledgments

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Korea. This study was also funded by a research grant from Biogreen 21 program (Code #200702157) and the BK21 project in Korea.

References

1. Benno Y, Mitsuoka T. Impact of *Bifidobacterium longum* on human

fecal microflora. Microbiol Immunol. 36: 683-694 (1992)
2. Fernández MF, Boris S, Barbés C. Probiotic properties of human *lactobacilli* strains to be used in the gastrointestinal tract. J. Appl. Microbiol. 94: 445-449 (2003)
3. Fooks LJ, Fuller R, Gibson GR. Prebiotics, probiotics, and human gut microbiology. Int. Dairy J. 9: 53-61 (1999)
4. Fuller R. Probiotics in human and animals. J. Appl. Bacteriol. 66: 365-378 (1989)
5. O'Sullivan GC. Probiotics. Brit. J. Surg. 88: 161-162 (2001)
6. Temmerman R, Pot B, Huys G, Swings J. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81: 1-10 (2003)
7. Isolauri E, Arvola T, Sütas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin. Exp. Allergy 30: 1604-1610 (2000)
8. Jayaprakasha HM, Yoon YC, Paik H-D. Probiotic functional foods and health claims: An overview. Food Sci. Biotechnol. 14: 523-528 (2005)
9. Shin MS, Kim YJ, Bae HS, Baek YJ. Effects of the lactic acid bacteria administration on fecal microflora and putrefactive metabolites in healthy adults. Korean J. Microbiol. Biotech. 24: 254-260 (1996)
10. Sullivan Å, Nord CE. Probiotics and gastrointestinal disease. J. Intern. Med. 257: 78-92 (2005)
11. Valraedas MMC, van der Mei HC, Reid G, Busscher JH. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolated. Appl. Environ. Microb. 62: 1958-1963 (1996)
12. Xanthopoulos V, Litopoulou-Tzanetaki E, Tzanetakis N. Characterization of *Lactobacillus* strains from infant faeces as dietary adjuncts. Food Microbiol. 17: 205-215 (2000)
13. Çaglar E, Kargul B, Tanboga I. Bacteriotherapy and probiotics' role on oral health. Oral Dis. 11: 131-137 (2005)
14. Vesa T, Pochart P, Marteau P. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharm. Therap. 14: 823-828 (2000)
15. Lee N-K, Jun S-A, Ha J-U, Paik H-D. Screening and characterization of bacterioncinogenic lactic acid bacteria from *jeotgal*, a Korean fermented fish food. J. Microbiol. Biotechn. 10: 423-428 (2000)
16. Lee NK, Kim HW, Chang HI, Yun CW, Kim SW, Kang CW, Paik H-D. Probiotic properties of *Lactobacillus plantarum* NK181 isolated from *jeotgal*, a Korean fermented food. Food Sci. Biotechnol. 15: 227-231 (2006)
17. Kobayashi Y, Tohyama K, Terashima T. Tolerance of the multiple antibiotic resistant strains, *L. casei* PSR 3002, to artificial digestive fluids. Jpn. J. Microbiol. 29: 691-697 (1974)
18. Lee SH, No MJ. Viability in artificial gastric and bile juice and antimicrobial activity of some lactic acid bacteria isolated from *kimchi*. J. Microbiol. Biotechn. 6: 617-622 (1997)
19. Singh PN, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191 (1988)
20. Hutkins RW, Nannen NL. pH homeostasis in lactic acid bacteria. J. Dairy Sci. 76: 2354-2365 (1993)
21. Kim JT, Jung HY, Lee NK, Rhim SL, Paik H-D. Isolation, identification, and probiotic properties of *Lactobacillus reuteri* HY701 from human feces. Food Sci. Biotechnol. 15: 677-682 (2006)
22. Vinderola CG, Reinheimer JA. Lactic acid starter and probiotic bacteria: A comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. Food Res. Int. 36: 895-904 (2003)
23. Cole CB, Fuller R, Carter SM. Effect of probiotic supplements of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* 2204 on β-glucosidase and β-glucuronidase activity in the lower gut of rats associated with a human fecal flora. Microb. Ecol. Health D. 2: 223-225 (1989)
24. Danielsen M, Wind A. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. 82: 1-11 (2003)
25. Pereira DIA, Gibson GR. Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Appl. Environ. Microb. 68: 4689-4693 (2002)
26. Clancy R. Immunobiotics and the probiotic evolution. FEMS Immunol. Med. Mic. 38: 9-12 (2003)