Characterization of Lactic Bacterial Strains Isolated from Raw Milk

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ABSTRACT : During lactic acid bacteria (LAB) transit through the gastrointestinal tract, ingested microorganisms were exposed to successive stress factors, including low pH in the human stomach and in bile acid. These stress factors can be used as criteria for the selection of a viable probiotic strain. Four such strains (*Lactobacillus helveticus* SGU 0011, *Lactobacillus pentosus* SGU 0010, *Streptococcus thermophilus* SGU 0021 and Lactobacillus *casei* SGU 0020) were isolated from raw milk. When the identified LAB were exposed to synthetic gastric juice, whereas *L. casei* SGU 0020 and *S. thermophilus* SGU 0021 exhibited a 0% survival rate, *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 exhibited 60% and 95% survival rates. *L. casei* SGU 0020 and *S. thermophilus* SGU 0021 could not be examined with regard to their tolerances to artificial bile juice, as they uniformly died upon exposure. However, *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 individually survived at rates of 39% and 93%. Also, all four of these strains were confirmed to be tolerant of ten different antibiotics. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 1 : 131-136)***

Key Words : Lactobacillus, Probiotics, Artificial Gastric Juice, rDNA, Antibiotic

INTRODUCTION

Today's consumers are increasingly aware of the importance of the maintenance of their environment, health and nutrition. In recent years, lactic acid bacteria, commercially used in dairy products including yoghurt, kimchi, and other fermented foods, has become well known as probiotics. Lactic acid bacteria generate lactic acid from carbohydrates, thus contributing to the pH reduction, flavor and other characteristics associated with a host of dairy products (Lilly, et al., 1965). The term "probiotic" was first introduced by Lilly and Stillwell (1965), to describe substances produced by one microorganism which stimulated the growth of other microorganisms. Since then, the term "probiotics" has undergone several modifications with regard to definition, the most common definition currently used being that provided by Fuller (1989). Fuller defined probiotics as live microbial feed supplements which exert beneficial effects on the host animal by improving its intestinal microbial balance (1989). During the transit of lactic acid bacteria through the gastrointestinal tract, ingested microoganisms were exposed to a series of stress factors, including low pH, in the human stomach and in bile acid (Simon et al., 1729; Marteau et al., 1993). Characteristics for the identification of probiotic bacteria have not been established. However, tests for bile tolerance, gastric acid tolerance and adherence to the mucosal surfaces of the host constitute several reasonable screening parameters for the selection of probiotic strains in nonruminant livestock (Pedersen et al., 2004).

The purpose of this study was to isolate the LAB from raw milk produced in Korea and to evaluate the potential effects of LAB as a probiotic strain by characterizing its acid and bile tolerances as well as its susceptibility to antibiotics *in vitro*.

MATERIALS AND METHODS

Bacterial strains and isolation

Raw milk obtained from the Sunhwa Dairy Farm (Suwon, Korea) and stored at 5°C. In order to isolate LAB, raw milk was cultured in *Lactobacillus* MRS broth, then spread onto Bromocrezol Purple Agar (BCP agar). After it was incubated at 37°C for 48 h, under either aerobic or anaerobic conditions, the colony were subcultured more than 3 times in Lactobacillus MRS agar. And then it was dissolved in skim milk solution containing 20% glycerol and stored at -70°C for further use. Each experiment used a stock freezer vial for medium inoculation

Identification by API kit and 16S rDNA sequencing

Strain species were identified according to previously recommended methods including bacteria morphology, gram staining, carbohydrate fermentation patterns and 16S rDNA sequencing (Holt et al., 1994). Therefore, the strains which had previously been isolated by pure culture were identified with an API 50 CHL Carbohydrate Test Kit (bioMerieux Co., France). These tests were conducted according to the instructions of the manufacturer, and by the database provided by bioMerieux. The isolated strains, after

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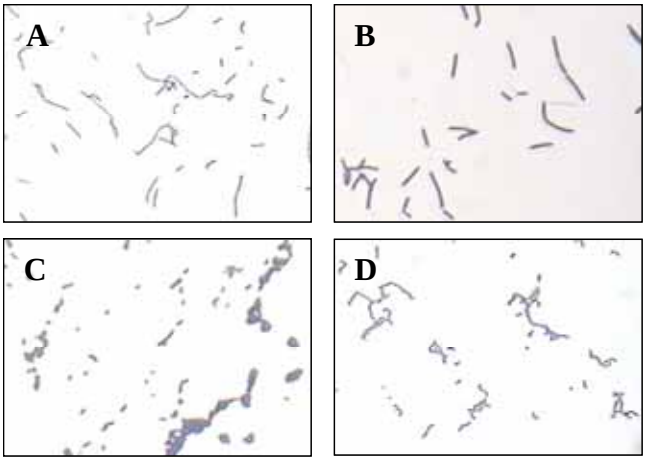

Figure 1. Results of gram staining under a light microscopy of the isolated strains from raw milk. A: *Lactobacillus casei* SGU 0020, B: *Lactobacillus helveticus* SGU 0011, C: *Lactobacillus pentosus* SGU 0010, D: *Streptococcus thermophilus* SGU 0021.

identification with an API kit, were subjected to 16S rDNA sequencing. After the DNA had been segregated from the strains treated with RNase, chloroform, and EtOH, it was amplified with PCR premix (Bioneer Co.) The forward primer used 5' -GA GTT TGA TCC TGG CTC AG -3'(in E. coli 16S rRNA no. position 9 to 27) and the reverse primer used 5‘-GGT TAC CTT GTT ACG ACT T -3' (in *E.coli* 16S rRNA no. 1492 to 1510). The amplified 16S rDNA was then purified with the AccuPrep PCR Purification Kit (Bioneer Co). The determination of the base sequences was carried out with a genetic analyzer 355 (Perkin-Elmer Co. USA) and was assessed by the CLUSTAL W program (Thompson, Germany) and the PHYLIP program (Felsenstein, USA).

Growth curve

It was important for us to ascertain the growth time of the LAB commercially used products in order to determine the stop point of fermentation. After 1% of the activated LAB had been inoculated into the *Lactobacilus* MRS broth, viable cell counts were assessed at 0, 6, 12, 24, 36 and 48 h. The viable cell numbers were counted after incubation at 37°C for 24 h.

Tolerance to artificial gastric juice

Before reaching the intestinal tract, the probiotic bacteria must first transit through the stomach (Henriksson et al., 1999). Therefore, tolerance to artificial gastric juice constitutes the basic conditions of probiotic bacteria. Tolerance to artificial gastric juice was assessed according to the method described by Kobayashi et al. (1974). The *Lactobacillus* MRS broth was adjusted to pH 2 with HCl and then was sterilized for 15 minutes at 121°C and was added to 1% pepsin. After the isolated LAB was subcultured at least 2 times, the LAB was added to it. After this mixture had been incubated for 1 h at 37°C, we performed plate counts using MRS agar and the pour plate technique on before and after incubation. Survival rates were then determined by evaluating changes of the C.F.U. (colony-forming units) for 1 h.

Tolerance to artificial bile acid

After the isolated LAB from raw milk was tested with regard to its tolerance to artificial gastric juice, then it was tested with regard to its tolerance to artificial bile acid. After MRS broth containing 0.1% oxgall was sterilized at 121°C for 15 minutes, 1% pancreatin was added to the medium. The LAB, which had been subcultured at least twice, was inoculated into the medium. After it had been incubated at 37°C for 1 h, plate counts were performed using MRS agar and the pour plate technique on before and after incubation. Survival rates were determined by evaluating changes of the C.F.U. (colony-forming units) for 1 h.

Antibiotic susceptibility tests

We used the disc diffusion method for test of susceptibility against antibiotics of the isolated LAB. 100 microliters of each activated strain was spread onto MRS plates and antibiotic discs impregnated with antibiotics were positioned on the MRS agar, and then they were incubated for 24 h at 37°C. We then measured the sizes of the resultant inhibitory zones. The antibiotic discs were subjected to testing with the Senti-disc^{TM} system (BBL Co., USA). The antibiotic names which used in this study were ampicillin (10 μg), carbenicillin (100 μg), erythromycin (15 μg), gentamicin (10 μg), lincomycin (2 μg), neomycin (30 μg), penicillin (10 IU/IE/UI), streptomycin (10 μg), sulfosoxazole (0.25 μg) and tetracycline (30 μg).

Statistical analysis

Significant differences between the experimental and control groups were determined using AVOVA (SAS ver. 8.2, USA). The results were expressed as mean±SD. The values of $p<0.05$ were considered significantly.

RESULTS AND DISCUSSION

The isolation and characterization of LAB

The selected LAB, which was isolated from the Sunhwa Dairy Farm (Suwon, Korea), was identified with the API 50 CHL Carbohydrate Test Kit (bioMerieux Co., France). Our four strains were identified by this system as: *Lactobacillus casei* SGU 0020, *Lactobacillus helveticus* SGU 0011, *Lactobacillus pentosus* SGU 0010 and *Streptococcus thermophilus* SGU 0021 (data, not presented).

After the isolated LAB was identified with the API kit,

Table 1. Result identified by 16S rDNA of selected strains from raw milk

API kit (50 CHL)	Identification (%)
Lactobacillus casei SGU 0020	98.15
Lactobacillus helveticus SGU 0011	99.76
Lactobacillus pentosus SGU 0010	99.90
Streptococcus thermophilus SGU 0021	99.84

we carried out 16S rDNA sequencing on the strains. The isolated strains identified that *Lactobacillus casei* SGU 0020 were 98.15% similar to *Lactobacillus casei* ATCC 25599^T and *L. helveticus* SGU 0011 was 99.76% similar to *Lactobacillus helveticus str.* Lh12 NCDO 2712^T and *L. pentosus* SGU 0010 were 99.9% similar to *Lactobacillus pentosus* JCM 1588 and *S. thermophilus* SGU 0021 was 99.84% similar to *Streptococcus thermophilus* ATCC 19258^T (Table 1). As the four strains isolated from the raw milk were more than 99% similar, we have concluded that the isolated strains were constituted a pure strain. We observed that the relationship between the results of the API 50 CHL Carbohydrate Test Kit and the 16S rDNA sequencing were remarkably consistent.

Growth curve

During the incubation of the LAB, we monitored the viable cell count. Our results revealed that *L. casei* SGU 0020 and *L. helveticus* SGU 0011 achieved stationary phase until 12 h after incubation and *L. pentosus SGU* 0010 was at 24 h and *S. thermophilus* SGU 0021 was at 36 h (Figure 2). The addition of LAB decreased the pH values in all cultures consistent with the known characteristics of LAB.

Tolerance to artificial gastric juice

The four strains were evaluated with regard to their tolerance to artificial gastric juice and the results of these evaluations are shown in Figure 3. The time necessary for food to pass through the human stomach is, in general, about 20-90 minutes (Renner, 1991). We set a fixed time of 60 minutes to represent transit time of food through a human stomach, the environmental conditions of which were simulated by adjusting the pH 2. After the identified LAB strains were exposed to artificial gastric juice for 1 h at 37°C, we carried out plate counts using MRS agar on before and after incubation. Whereas *L. casei* SGU 0020 and *S. thermophilus* SGU 0021 exhibited 0% survival rates, *L. helveticus* SGU 0011 had a 60% survival rate and *L. pentosus* SGU 0010 had a 95% survival rate. *L. pentosus* SGU 0010 exhibited the highest survival rate among the four strains. According to Ronka et al. (2003), *L. brevis* evidenced a 78% survival rate in the artificial gastric juice with pH 2 after 1 h. And Ingrid (2003) and Usman (2003)

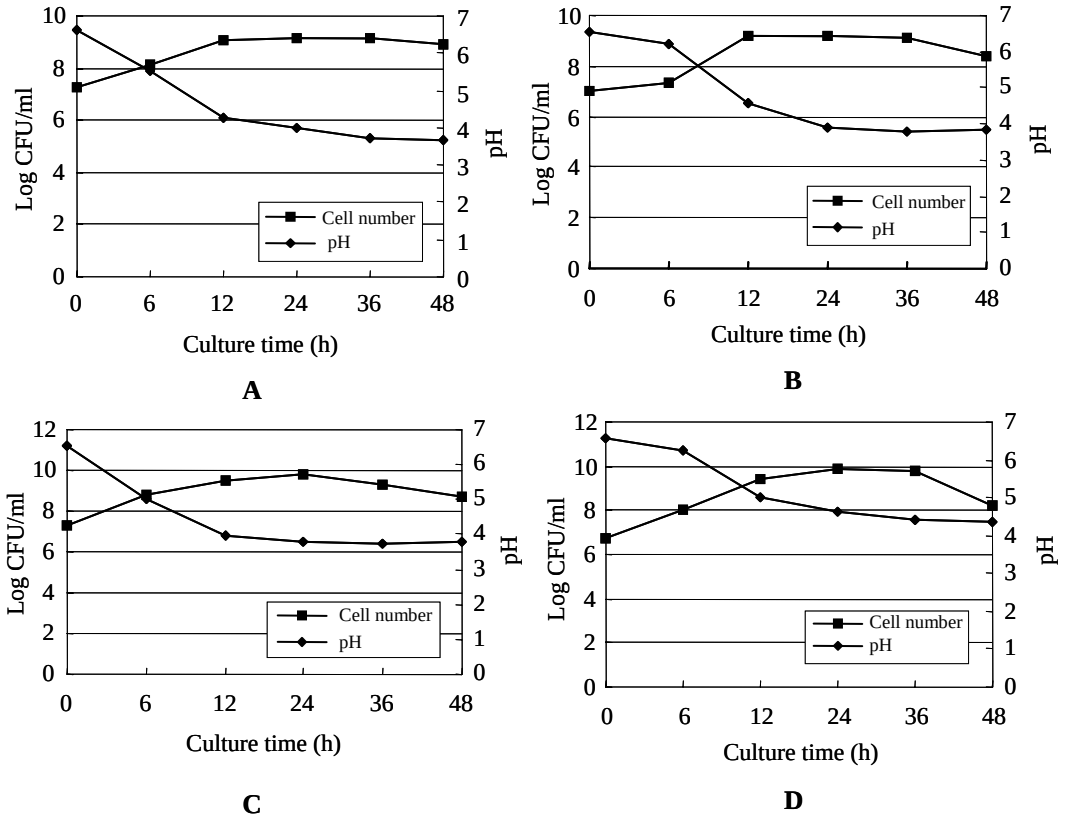

Figure 2. Growth patterns and pH of isolated LAB in MRS broth at 37°C for 48 h. A: *Lactobacillus casei* SGU 0020, B: *Lactobacillus helveticus* SGU 0011, C: *Lactobacillus pentosus* SGU 0010, D: *Streptococcus thermophilus* SGU 0021.

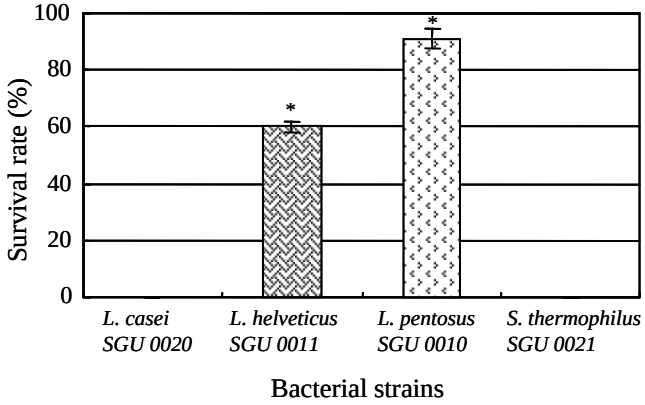

Figure 3. Survival rate of strains isolated from raw milk in artificial gastric juice. * Means in a given column with different superscript are significantly different (*p<0.05). The *Lactobacillus* MRS broth was adjusted to pH 2 with HCl and then was sterilized for 15 minutes at 121°C and was added to 1% pepsin. After the isolated LAB was subcultured at least 2 times, the LAB was added to it. After this mixture had been incubated for 1 h at 37°C, we performed plate counts using MRS agar and the pour plate technique on before and after incubation. Survival rates were then determined by evaluating changes of the CFU (colony-forming units) for 1 h.

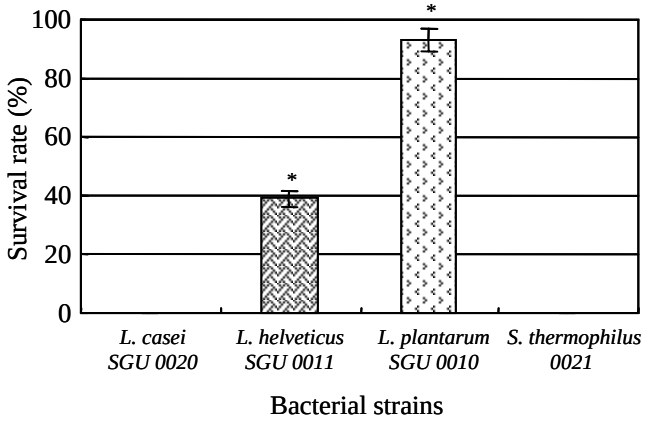

Figure 4. Survival rate of strains isolated from raw milk in artificial bile acid. * Means in a given column with different superscript are significantly different (* p<0.05). After MRS broth containing 0.1% oxgall was sterilized at 121°C for 15 minutes, 1% pancreatin was added to the medium. The LAB, which had been subcultured at least twice, was inoculated into the medium. After it had been incubated at 37°C for 1 h, plate counts were performed using MRS agar and the pour plate technique on before and after incubation. Survival rates were determined by evaluating changes of the CFU (colony-forming units) for 1 h.

revealed that 10 strains of LAB had a 50-80% survival rate. The tolerance of LAB to acid appears to depend on the pH profile of its H^+-ATPase, as well as the composition of its cytoplasmic membrane, which is largely influenced by the type of bacteria, type of media, and incubation conditions (Hood et al., 1988; Havenaar et al., 1992). Some of these strains were able to pass through the stomach without undergoing significant losses in viability (Robins-Browne et al., 1981; Havenaar et al., 1992). As there was a clear correlation between the *in vivo* and *in vitro* experiments, the acid tolerance test with the artificial gastric juice is commonly used to select viable probiotic strains.

Tolerance to artificial bile acid

We constructed a simulated digestive organ for the *in vivo* tests and the LAB strains were exposed to artificial gastric juice for 1 h at 37°C. The viable cell counts were then assessed for their tolerance to artificial bile acid. Our results are shown in Figure 4. *L. casei* SGU 0020 and *S. thermophilus* SGU 0021 were not included in these tests, as all of these bacteria perished in the artificial gastric juice. *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 individually survived at rates of 39% and 93%.

Bile tolerance is one of the most crucial criteria for any strain to be used as a probiotic culture (Dunne et al., 2001). Bile salts are surface-active chemicals which are produced in the liver by the catabolism of cholesterol. Bile acid consists of chenodeoxycholic acid, cholic acid, deoxycholic acid and other minor components secreted from the spleen into the duodenum of the small intestine (Brandt et al., 1976). Bile acids have been demonstrated to inhibit microorganisms and their inhibitory activity is greater than that of the organic acids (Robins-Browne et al., 1981). The deconjugated forms tend to be more inhibitory and gram-positive bacteria tend to be more sensitive than gram-negative bacteria (Floch et al., 1972; Stewart et al., 1986).

Antibiotic susceptibility tests

The susceptibility to antibiotics became generally known the basic conditions of probiotic bacteria. The susceptibility of the isolated LAB strains to antibiotics was assessed via the disc diffusion method. According to the M2-A4 (Performance Standards for Antimicrobial Disk Susceptibility Test-Fourth Edition; Approved Standard) confirmed NCCLS, susceptibility to antibiotics is graded as either R (Resistant) or S (Susceptible) (Oh et al., 2000) and our results for these tests are shown in Table 2. All four of our isolated strains were confirmed to exhibit tolerance to ten different antibiotics. The four strains did not exhibit no inhibitory zones against 0.25 microgram of sulfosoxazole, but it did exhibit inhibitory zones under 2.5 mm in diameter against 10 μg of gentamycin, 30 μg of neomycin and 10 μg of streptomycin. *S. thermophilus* SGU 0021 was confirmed to be the most susceptible to antibiotics of the isolated LAB strains.

Summary

Four strains (*Lactobacillus casei* SGU 0020, *Lactobacillus helveticus* SGU 0011, *Lactobacillus pentosus* SGU 0010 and *Streptococcus thermophilus* SGU 0021)

Table 2. Antibiotic susceptibility of isolated strains from raw milk (Unit: mm)

Antibiotic \ Strains	*L. casei* SGU 0020	*L. helveticus* SGU 0011	*L. pentosus* SGU 0010	*S. thermophilus* SGU 0021
Ampicillin (10 μg)	R(9)	R(10)	R(11)	R(10)
Carbenicillin (100 μg)	R(9)	R(13)	R(8)	R(10)
Erythromycin (15 μg)	R(-)	R(-)	R(6)	R(7)
Gentamicin (10 μg)	R(2)	R(2.5)	R(2)	R(1)
Lincomycin (2 μg)	R(9)	R(1)	R(7)	R(3)
Neomycin (30 μg)	R(2)	R(2)	R(2.5)	R(-)
Penicillin (10 IU/IE/UI)	R(8)	R(11)	R(5)	R(7)
Streptomycin (10 μg)	R(1)	R(6)	R(1)	R(-)
Sulfosoxazole (0.25 μg)	R(-)	R(-)	R(-)	R(-)
Tetracycline (30 μg)	R(8)	R(12)	R(3)	R(8)

* R: Resistant, S: Susceptible.

were isolated from the Sunhwa Dairy Farm (Suwon, Korea). During lactic acid bacteria (LAB) transit through the gastrointestinal tract, ingested microorganisms were exposed to successive stress factors, including low pH in the human stomach and in bile acid. These stress factors can be used as criteria for the selection of a viable probiotic strain. All four of these strains were confirmed to be tolerant of ten different antibiotics. But, whereas *L. casei* SGU 0020 and *S. thermophilus* SGU 0021 exhibited a 0% survival rate, *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 exhibited 60% and 95% survival rates. *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 individually survived at rates of 39% and 93%. *L. helveticus* SGU 0011 and *L. pentosus* SGU 0010 can be utilized commercially as probiotic strain on health functional food and milk fermentation.

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REFERENCES

Brandt, L. J. and L. H. 1976. Bernstein. Bile salts: their role in cholesterol synthesis, secretion and lithogenesis. Am. J. Gastroenterol. 65:17-30.

Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan and J. K. Collins. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am. J. Clin. Nutr. 73:386S-392S.

Floch, M. H., H. J. Binder, B. Filburn and W. Gershengoren. 1972. The effect of bile acids on intestinal microflora. Am. J. Clin. Nutr. 25:1418-1426.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gilliland, S. E. 1979. Beneficial interrelationships between certain microoganisms and humans: candidate microoganism for use as dietary adjuncts. J. Food Prot. 42:164-167.

Havenaar, R., B. T. Brink and J. H. J. Huis isn't Veld. 1992. Selection of strains for probiotic use. pp. 209-224 in probiotics. (Ed. R. Fuller). Chapman & Hall. London.

Henriksson, A., A. K. D. Khaled and P. L. Conway. 1999. *Lactobacillus* colonization of the gastrointestinal tract of mice after removal of the nonsecreting stomach region. Microb. Ecol. Health Dis. 11:96-99.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams. 1994. In: Bergey's Manual of Determinative Bacteriology, 9th Ed. (Ed. W. R. Hensyl), pp. 527-566. Williams and Wilkins, Baltimore, MD.

Hood, S. K. and E. A. Zottola. 1988. Effects of low pH on the ability of *Lactobacillus acidophillus* to survive and adhere to human intestinal cells. J. Food Sci. 53:1514-1516.

Ingrid, S. Surono. 2003. *In vitro* probiotic properties of indigenous dadih lactic acid bacteria. Asian-Aust. J. Anim. Sci. 16(5):726-731.

Kobayashi, Y., K. Toyama and T. Terashima. 1974. [Biological characteristics of *Lactobacillus.* II. Tolerance of a multiple antibiotic resistant strain, *Lactobacillus casei* PSR 3002, to artificial digestive fluids] Nippon Saikingaku Zasshi. 29:691-697.

Lilly, D. M. and R. H. Stillwell. 1965. Probiotics: growth-promoting factors produced by microorganisms. Sci. 147:747-748.

Marteau, P., P. pochart, Y. Bouhnik and J. C. Rambaud. 1993. Fate and effects of some transiting microoganism in the human gastrointestinal tract. World Reu. Nutr. Diet. 74:1.

Oh, S., S. H. Kim and R. W. Worobo. 2000. Characterization and purification of a bacteriocin produced by a potential probiotic culture, Lactobacillus acidophilus 30SC. J. Dairy Sci. 83:2747-2752.

Pedersen, C., H. Jonsson, J. E. Lindberg and S. Roos. 2004. Microbiological characterization of wet wheat distillers' grain, with focus on isolation of lactobacilli with potential as probiotics. Appl. Environ. Microbiol. 70:1522-1527.

Renner, E. 1991. Cultured dairy products in human nutrition. Bull. Int. Dairy Fed. 255:2-24.

Robins-Browne, R. M. and M. M. Levine. 1981. The fate of ingested Lactobacilli in the proximal small intestine. Am. J. Clin. Nutr. 34:514-519.

Ronka, E., E. Malinen, M. Sarela, M. Rinta-Koski, J. Aarnikunnas

and A. Palva. 2003. Probiotic and milk technological properties of *Lactobacillus brevis*. Int. J. Food Microbiol. 83:63-74.

Simon, G. L. and S. L. Gorbach. 1987. Intestinal flora and gastrointestinal function. pp. 1729 in Physiology of the gastrointestinal tract. Vol. 2. 2nd (Ed. L. R. Johnson), ed Raven Press, New York.

Stewart, L., C. A. Pellegrini and L. W. Way. 1986. Antibacterial activity of bile acids against common biliary tract organism. Surg Forum. 37:157-159.

Usman Pato. 2003. Bile and acid tolerance of lactic acid bacteria isolated from dadih and their antimutagenicity against mutagenic heated tauco. Asian-Aust. J. Anim. Sci. 16(11):1680-1685.