

Characterization of Selected *Lactobacillus* Strains for Use as Probiotics

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Abstract

The aim of this study was to evaluate the functional properties of lactic acid bacteria from various sources and to identify strains for use as probiotics. Ten *Lactobacillus* strains were selected and their properties such as bile tolerance, acid resistance, cholesterol assimilation activity, and adherence to HT-29 cells were assessed to determine their potential as probiotics. *Lactobacillus* sp. JNU 8829, *L. casei* MB3, *L. sakei* MA9, *L. sakei* CH8, and *L. acidophilus* M23 were found to show full tolerance to the 0.3% bile acid. All strains without *L. acidophilus* M23 were the most acid-tolerant strains. After incubating the strains at pH 2.5 for 2 h, their viability decreased by 3 Log cells. Some strains survived at pH 2.5 in the presence of pepsin and 0.3% bile acid. *Lactobacillus* sp. JNU 8829, *L. acidophilus* KU41, *L. acidophilus* M23, *L. fermentum* NS2, *L. plantarum* M13, and *L. plantarum* NS3 were found to reduce cholesterol levels by >50% *in vitro*. In the adhesion assay, *Lactobacillus* sp. JNU 8829, *L. casei* MB3, *L. sakei* MA9, and *L. sakei* CH8 showed higher adhesion activities after 2 h of co-incubation with the intestinal cells. The results of this comprehensive analysis shows that this new probiotic strain named, *Lactobacillus* sp. JNU 8829 could be a promising candidate for dairy products.

Keywords: *Lactobacillus*, probiotics, acid and bile acid tolerances, cholesterol

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Introduction

As recently outlined in the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) expert consultation on the evaluation of health and nutritional properties of probiotics in food, including milk powder with live lactic acid bacteria (LAB), probiotic strains are defined as live microorganisms that, when consumed in an adequate amount as part of food, provide a health benefit to the host (Servin, 2003). Probiotics are commonly used as feed supplements that exert their beneficial effects by improving the intestinal microbial balance of the host animal. Probiotics used for animals include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, and *Enterococcus* strains (Hyronimus

et al., 2000). LAB include native microorganisms residing in the GI tracts of diverse animals. They are believed to play a leading part in the health of the host. The relationship between resident LAB and the host animal and the role of colonization of the GI tract by LAB in promoting the health of the host have garnered attention (Miyoshi *et al.*, 2006).

LAB such as *Lactobacillus* and *Bifidobacterium* spp. have been reported to exert health promoting or 'probiotic' effects in humans and animals (du Toit *et al.*, 1998). Factors such as bile tolerance, which enable a selected strain to survive, grow, and exert therapeutic benefits in the intestinal tract should also form selection criteria for the evaluation of *Lactobacillus* strains as candidates in the development of probiotic food supplements (Usman and Hosono, 1999).

The probiotic effects of LAB are reported to involve inhibition of pathogenic microorganisms, protection against gastrointestinal diseases, anti-mutagenic and anti-carcinogenic activities, and enhancement of the host immune response (du Toit *et al.*, 1998; Nguyen *et al.*, 2007). The reduction of cholesterol by LAB has also been demonstrated

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in humans, mice, and pigs. These cholesterol-lowering effects may be attributed, in part, to the deconjugation of bile acid salts by strains of bacteria that produce the enzyme bile acid salt hydrolase (BSH), as well as short-chain fatty acid fermentation, and cholesterol binding to the bacterial cell wall (Brashears *et al.*, 1998; Pereira and Gibson, 2002). As deconjugated bile acid salts are more readily excreted in the feces than conjugated bile acid salts, bacteria with BSH activity may reduce serum cholesterol by enhancing the excretion of bile acid salts, with a consequent increase in the synthesis of bile acid salts from serum cholesterol, or by decreasing the solubility of cholesterol, and thus reducing its uptake from the gut (Nguyen *et al.*, 2007).

The objectives of this study were to evaluate acid resistance, bile tolerance, adherence to HT-29 cells, and cholesterol-lowering ability of several *Lactobacillus* strains and to identify potentially useful candidate probiotic *Lactobacillus* strains.

Materials and Methods

Selection of *Lactobacillus* strains

Table 1 shows the selected *Lactobacillus* strains. The strains were isolated from kimchi, dairy products, fermented olive and fecal matter from a Korean infant. For the selection of lactobacilli, all samples were cultured on de Man, Rogosa, and Sharpe (MRS) agar (Difco, USA) then acidified to pH 5.4 at 37°C for 48 h. All isolates were characterized as *Lactobacillus* based on Gram staining, catalase test and their ability to change color on the bromocresol purple agar. The final confirmation of their identity was achieved by the 16S rDNA sequence analysis. Ten strains of *Lactobacillus* were inoculated on plates of MRS agar and incubated for 48 h at 37°C. Cultures were maintained in 10% skim milk with 1% glucose and 0.3% yeast extract, and stored at -80°C.

Acid and bile tolerance

Acid and bile tolerance was tested using the method described by Hyronimus *et al.* (2000). Acid tolerance of cultures were grown in MRS broth at 37°C for 18 h and subcultured in 10 mL of fresh MRS broth, adjusted to pH 2.5 with 1,000 units/mL pepsin (Sigma Co., USA). The initial bacterial concentration was 1.0×10^8 CFU/mL, and samples were incubated for 0 and 2 h at 37 °C. Cultures (1.0×10^8 CFU/mL) were serially diluted 10-fold in 0.05 M sodium phosphate buffer (pH 7.0) to neutralize medium acidity. In the assay of bile tolerance, cells were grown in

Table 1. Selected strains of *Lactobacillus* used in this study

Strains	Origin
<i>Lactobacillus</i> sp. JNU 8829	Infant feces
<i>Lactobacillus acidophilus</i> KU41	Infant feces
<i>Lactobacillus acidophilus</i> M23	Infant feces
<i>Lactobacillus brevis</i> CH7	Kimchi
<i>Lactobacillus casei</i> MB3	Kimchi
<i>Lactobacillus fermentum</i> NS2	Kimchi
<i>Lactobacillus plantarum</i> M13	Kimchi
<i>Lactobacillus plantarum</i> NS3	Kimchi
<i>Lactobacillus sakei</i> CH8	Fermented olive
<i>Lactobacillus sakei</i> MA9	Fermented olive

MRS broth at 37°C for 18 h and then re-cultured in 10 mL of fresh MRS broth containing 0.3% (w/v) of oxgall at 37°C. Bile tolerance was then determined by comparing the final plate count after 24 h regarding the initial plate count at 0 h. Each experiment was conducted in triplicate.

Cholesterol assimilation

The cholesterol assimilation assay was performed according to the method described by Buck and Gilliland (1994). The strains were inoculated into MRS broth (polyoxyethanyl cholesteryl sebacate 0.045% and cysteine 0.05%) for 24 h under anaerobic conditions and then centrifuged (12,000 g, 10 min, 4°C). The supernatant was recovered, and the amount of cholesterol remaining in it was determined. The cholesterol content of the supernatant was determined using a modified colorimetric method as previously described by Rudel and Morris (1973). A total of 0.5 mL supernatant fluid was collected and was mixed with 2 mL KOH (50%, w/v) and 3 mL 97% ethanol and then reacted in a water bath at 60°C for 5 min. After cooling, the cells were added to 5 mL hexane and mixed. A 3 mL aliquot of distilled water was added, mixed, and clean tubes were allowed to stand for 15 min at room temperature to allow for phase separation. A 2.5 mL aliquot of hexane layer fluid was transferred to a new tube, and the hexane was evaporated under nitrogen gas. After the remaining solution was treated with 4 mL of o-phthalaldehyde reagent (0.5 mg o-phthalaldehyde/glacial acetic acid 1 mL), 2 mL of sulfuric acid was added and reacted for 10 min. The results were monitored by measuring absorbance with a microplate reader (Synergy HT, Bio-Tek, USA) at 550 nm. Each experiment was conducted in triplicate.

$$\text{Cholesterol reduction (\%)} = \frac{(\text{Cholesterol added}) - (\text{Cholesterol left})}{\text{Cholesterol added}} \times 100$$

Adhesion assay

Assays to determine adhesion were performed using the method of Kim *et al.* (2008) with slight modifications. The monolayers of HT-29 cells (KCTC, Korea) were washed five times in PBS and overlaid with 0.5 mL of RPMI 1640 medium (Gibco BRL, USA). A total of 1.0×10^9 CFU/mL of strains in antibiotic-free medium were mixed, inoculated to each well, and then incubated for 0 and 24 h at 37°C. The monolayer cells were washed 3 times in PBS to remove any unattached bacteria. The adherent cells were released from well plates using 0.2% trypsin-EDTA and incubated for 5 min at 37°C in 5% CO₂-95% air atmosphere. The number of viable cells was determined using the spread plate method on MRS agar with incubation at 37°C for 48 h. Each experiment was conducted in triplicate.

Statistical analysis

All values are expressed as mean±standard error (SE). Data was analyzed by one-way ANOVA using SPSS ver. 18.0 (SPSS Inc., USA). Differences between groups were assessed using Duncan's multiple range test. Statistical significance was considered at $p < 0.05$.

Results and Discussion

Acid and bile tolerance

Similar pH tolerance was observed in all strains examined for significant differences in cell viabilities for 2 h, at low pH. All strains showed tolerance to pH 2.5 for 2 h despite variations in the degree of viability (Table 2). The nine strains without *L. acidophilus* M23 were most acid-tolerant strains after incubation at pH 2.5 for 2 h. Out of the nine cells, eight of them decreased by 3 log cells, but one *L. acidophilus* M23 strain showed decrease by 4.1 log cells. As some strains have numerous acid-shock proteins that promote survival, these are capable of surviving exposure to extreme acidic environments (Merrell and Camilli, 2002). These results are in agreement with other studies which demonstrate that *Lactobacillus* strains remain viable when exposed to pH values of 2.5-4.0, but exhibit loss of viability at lower pH values (du Toit *et al.*, 1998; Dunne *et al.*, 2001; Jacobsen *et al.*, 1999; Liong and Shah, 2005; Maragkoudakis *et al.*, 2006). The term 'probiotic' means 'for life', and describes microorganisms (in most cases, bacteria) that survive passage through the gastrointestinal tract and have beneficial effects on the host (Wang *et al.*, 2012). Acid-tolerant strains have an advantage in surviving in the low pH conditions of the

Table 2. Survival of isolated strains after 2 h in modified MRS broth

Strains	Pepsin at pH 2.5 (CFU/mL)	
	0 h	2 h
<i>Lactobacillus</i> sp. JNU 8829	$2.30 \times 10^8 \pm 0.07$	$1.76 \times 10^6 \pm 0.10$
<i>Lactobacillus acidophilus</i> KU41	$2.20 \times 10^8 \pm 0.01$	$1.70 \times 10^7 \pm 0.01$
<i>Lactobacillus acidophilus</i> M23	$1.60 \times 10^8 \pm 0.04$	$1.27 \times 10^4 \pm 0.02$
<i>Lactobacillus brevis</i> CH7	$2.00 \times 10^8 \pm 0.03$	$5.00 \times 10^7 \pm 0.08$
<i>Lactobacillus casei</i> MB3	$1.24 \times 10^7 \pm 0.02$	$4.50 \times 10^5 \pm 0.36$
<i>Lactobacillus fermentum</i> NS2	$8.15 \times 10^7 \pm 0.04$	$1.29 \times 10^6 \pm 0.16$
<i>Lactobacillus plantarum</i> M13	$3.53 \times 10^8 \pm 0.04$	$2.00 \times 10^7 \pm 0.22$
<i>Lactobacillus plantarum</i> NS3	$3.90 \times 10^7 \pm 0.02$	$7.70 \times 10^5 \pm 0.28$
<i>Lactobacillus sakei</i> CH8	$2.00 \times 10^7 \pm 0.03$	$1.90 \times 10^8 \pm 0.04$
<i>Lactobacillus sakei</i> MA9	$1.63 \times 10^7 \pm 0.07$	$1.50 \times 10^5 \pm 0.15$

Table 3. Effect of 0.3% bile concentration on the viability of isolated strains

Strains	0.3% oxgall (CFU/mL)	
	0 h	24 h
<i>Lactobacillus</i> sp. JNU 8829	$2.18 \times 10^6 \pm 0.02$	$4.45 \times 10^6 \pm 0.03$
<i>Lactobacillus acidophilus</i> KU41	$2.28 \times 10^6 \pm 0.01$	$3.46 \times 10^5 \pm 0.33$
<i>Lactobacillus acidophilus</i> M23	$1.07 \times 10^6 \pm 0.01$	$3.87 \times 10^6 \pm 0.01$
<i>Lactobacillus brevis</i> CH7	$6.32 \times 10^6 \pm 0.06$	$1.08 \times 10^7 \pm 0.01$
<i>Lactobacillus casei</i> MB3	$7.84 \times 10^6 \pm 0.02$	$6.65 \times 10^8 \pm 0.11$
<i>Lactobacillus fermentum</i> NS2	$1.36 \times 10^6 \pm 0.02$	$6.90 \times 10^3 \pm 0.01$
<i>Lactobacillus plantarum</i> M13	$7.98 \times 10^5 \pm 0.02$	$7.48 \times 10^2 \pm 0.02$
<i>Lactobacillus plantarum</i> NS3	$7.83 \times 10^5 \pm 0.02$	$3.09 \times 10^3 \pm 0.08$
<i>Lactobacillus sakei</i> CH8	$7.83 \times 10^5 \pm 0.01$	$1.63 \times 10^7 \pm 0.05$
<i>Lactobacillus sakei</i> MA9	$6.94 \times 10^6 \pm 0.21$	$4.20 \times 10^8 \pm 0.05$

stomach (as low as pH 2.0), where hydrochloric and gastric acids are secreted (du Toit *et al.*, 1998).

Lactic acid bacteria were isolated and analyzed for their probiotic properties, including acid and bile tolerance. The effects of oxgall on the growth of selected strains revealed *Lactobacillus* sp. JNU 8829, *L. acidophilus* M23, *L. brevis* CH7, *L. casei* MB3, *L. sakei* CH8 and *L. sakei* MA9 to all have full tolerance to 0.3% bile acid (Table 3). These results indicate that bile acid at 0.3% does not affect the viability of these 4 strains and that all isolates could grow in the presence of 0.3% bile. In general, the physiological concentration of human bile ranges from 0.3% to 0.5% (Dunne *et al.*, 2001). Therefore, resistance to bile acid is an important characteristic that enables *Lactobacillus* to survive, grow, and remain active in the small intestine (du Toit *et al.*, 1998; Hyronimus *et al.*, 2000). Most studies have reported that the majority of strains survived well under such conditions, suggesting a possible recovery of the initial cell number during passage through the small intestine (Charteris *et al.*, 1998; du Toit *et al.*, 1998; Jacobsen *et al.*, 1999). Chou and Weimer (1999) reported that although such studies have major differences

species in design, they all show that acid and bile acid have separate and combined effects on the bacterial growth. Therefore, the acid and bile tolerance demonstrated by the LAB studies here suggest that these strains are likely resistant to stomach and intestinal conditions.

Cholesterol assimilation

The cholesterol assimilation abilities of selected strains of *Lactobacillus* were investigated (Fig. 1). *Lactobacillus* sp. JNU 8829, *L. acidophilus* KU41, *L. acidophilus* M23, *L. fermentum* NS2, *L. plantarum* M13, and *L. plantarum* NS3 were found to reduce cholesterol levels by >50% in the *in vitro* test. *Lactobacillus sakei* MA9 and *L. sakei* CH8 found to decrease cholesterol by 30%, whereas *L. brevis* CH7 and *L. casei* MB3 decreased cholesterol slightly below 30%. These results suggest that selected strains were able to remove cholesterol *in vitro* by inhibiting the formation of cholesterol micelles with bile acids. Cholesterol assimilation by LAB in the gastrointestinal tract would enable the decrease of cholesterol absorption by enterocytes, promoting the excretion of the cholesterol from the host. Noh *et al.* (1997) suggested that *L. acidophilus* ATCC 43121 shown resistant greater resistance to lysis by sonication when grown in the presence of cholesterol micelles and bile acid salts. They concluded that this resistance may be due to cholesterol assimilation into the cellular membrane, resulting in sturdier bacterial cells (Noh *et al.*, 1997). Further, Kumar (2012) reported that incorporation of cholesterol micelles by probiotic bacterial cells in the gut may explain the hypocholesterolemic

effects exerted by these bacteria. In addition, Nilakhe and Sapre (2015) reported that *in vivo* hypocholesterolemic ability may be due to cholesterol assimilation by *L. acidophilus* cells, or attachment of cholesterol to their surface. Therefore, screening for cholesterol-lowering properties *in vitro* has become an important criterion in the selection of bacterial strains for *in vivo* probiotic investigations.

Adhesion assay

Adherence of different bacteria has been studied using eukaryotic cell culture as an *in vitro* model of the human intestinal mucosa (Lehto and Salminen, 1997). Adhesion and colonization at the intestinal surface may be important prerequisites for probiotic strains to exert beneficial effects in the large intestine (Lim, 2014). The selected strains of *Lactobacillus* were similarly studied for adhesion to HT-29 cells (Fig. 2). In our study, *Lactobacillus* sp. JNU 8829, *L. acidophilus* M23, *L. casei* MB3, *L. sakei* CH8, *L. sakei* MA9 showed higher adhesion abilities than other strains. The viable cell count of *Lactobacillus* sp. JNU 8829 was lower than that of *L. casei* MB3, but, compared with results of Kim *et al.* (2008), adhesion of *Lactobacillus* sp. JNU 8829 showed higher cell viability. Kim *et al.* (2007) reported that the main criterion in the evaluation of probiotic strains is their ability to adhere to the intestinal epithelial cell line. Therefore, adhesion of the probiotic strains to the intestinal mucosa is considered a prerequisite for successful colonization, and is crucial for antagonistic activity against enteric bacterial pathogens (Ouweland *et al.*, 1999).

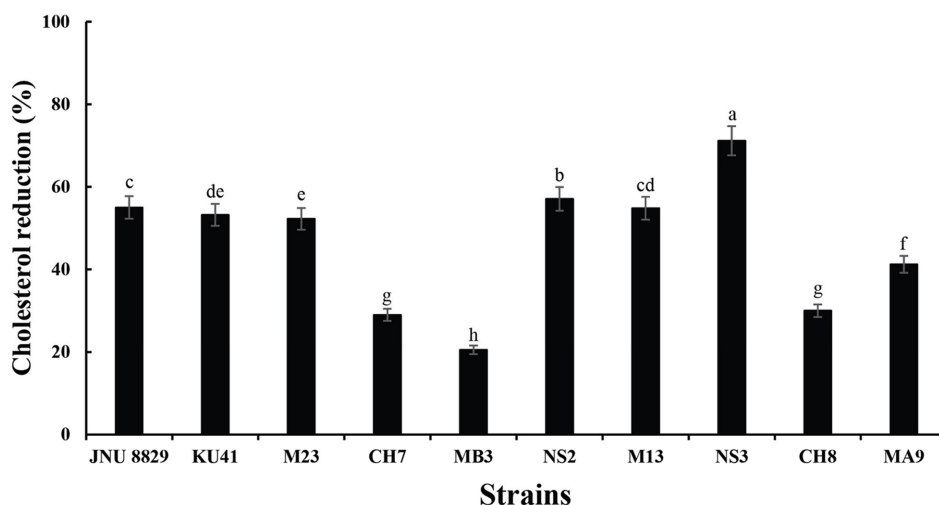


Fig. 1. Reduction of cholesterol by isolated strains *in vitro*. JNU 8829, *Lactobacillus* sp.; KU41, *L. acidophilus*; M23, *L. acidophilus*; CH7, *L. brevis*; MB3, *L. casei*; NS2, *L. fermentum*; M13, *L. plantarum*; NS3, *L. plantarum*; CH8, *L. sakei*; and *L. sakei* MA9. Each value is expressed as mean±SE. Values with different letters are significantly different ($p < 0.05$).

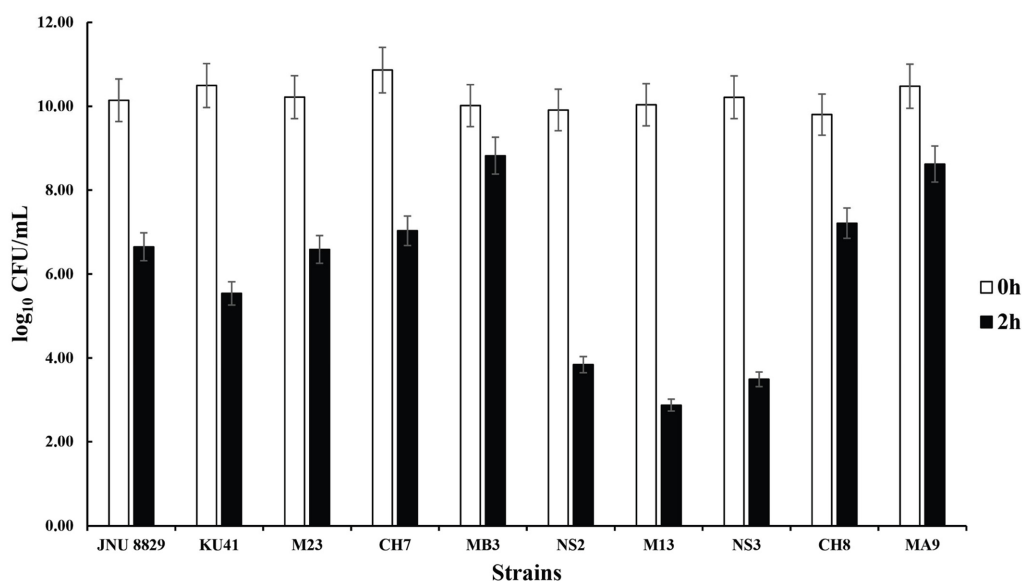


Fig. 2. Adherence of selected *Lactobacillus* strains to HT-29 cells. JNU 8829, *Lactobacillus* sp.; KU41, *L. acidophilus*; M23, *L. acidophilus*; CH7, *L. brevis*; MB3, *L. casei*; NS2, *L. fermentum*; M13, *L. plantarum*; NS3, *L. plantarum*; CH8, *L. sakei*; and *L. sakei* MA9. Each value is expressed as mean±SE.

Conclusion

This study was aimed evaluating, *in vitro*, several probiotic properties of selected LAB strains; namely their acid resistance, bile acid tolerance, cholesterol assimilation activity, and adhesion to cells. The strains evaluated demonstrated sufficient acid and bile tolerance to be resistant to physiological stomach and intestinal conditions, making them potentially useful candidates for the development of probiotics. Cholesterol assimilation by the five strains *Lactobacillus* sp. JNU 8829, *L. acidophilus* KU41, *L. acidophilus* M23, *L. fermentum* NS2, *L. plantarum* M13, and *L. plantarum* NS3 ranged from 52.25 to 71.16%. *Lactobacillus* sp. JNU 8829, *L. fermentum* NS2, and *L. plantarum* NS3 were found to have the highest levels of cholesterol assimilation. These selected *Lactobacillus* strains also showed adhesive abilities. The *Lactobacillus* sp. JNU 8829 strain was found to have the most potential effectiveness as a probiotic based on its acid and bile tolerance, cholesterol-lowering effects and adhesion to intestinal cells. Therefore, this strain would make a good candidate for further investigation through *in vivo* studies to determine its potential health benefits.

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