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Isolation, Identification, and Probiotic Properties of *Lactobacillus reuteri* HY701 from Human Feces

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Abstract Strain HY701 was isolated from human feces for probiotic use by selecting highly resistant isolates to artificial gastric acid and bile acid. Strain HY701 was identified as *Lactobacillus reuteri* using 16S rDNA sequencing, and tentatively named *L. reuteri* HY701. The resistance of *L. reuteri* HY701 to artificial gastric acid (pH 2.5) was high with a survival rate of over 90%. *L. reuteri* HY701 also showed high tolerance to artificial bile acid after incubation in artificial gastric acid. Using the API ZYM test kit, the carcinogenic enzymes β-glucuronidase and β-glucosidase were not detected with *L. reuteri* HY701, while the beneficial enzyme β-galactosidase was weakly detected. *L. reuteri* HY701 was sensitive to 100 μg/mL nisin, 20 μg/mL roxithromycin, 15 μg/mL erythromycin, but resistant to 20 μg/mL streptomycin, 10 μg/mL tetracycline, 20 μg/mL ciprofloxacin, 20 μg/mL nystatin, 20 μg/mL gentamycin, 10 μg/mL doxycycline, 10 μg/mL chloramphenicol, and 20 μg/mL ampicillin. *L. reuteri* HY701 was shown to possess bactericidal activity as it inhibited the growth of *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* JM109 completely within 24 hr of incubation. These results indicate that *L. reuteri* HY701 could be used as a probiotic strain.

Keywords: probiotics, *Lactobacillus reuteri* HY701, probiotic properties, *Listeria monocytogenes*

Introduction

Probiotics are generally defined as viable microorganisms that, when applied to humans or animals, beneficially affect the health of the host by improving the indigenous microbial balance (1, 2). Most probiotics belong to the large group of bacteria empirically designated as lactic acid bacteria (LAB; *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus*), which are important components of the human gastrointestinal microflora and exist as harmless commercial organisms.

Probiotics require the following characteristics to be effective: (i) genera of human origin; (ii) stability and ability to adhere to the intestinal mucosa (3); (iii) colonization potential in the human gastrointestinal tract; (iv) production of antimicrobial substances; and (v) demonstrable efficacy and safety. In addition, it is important that the viability of the strain and stability of its desirable characteristics are maintained during commercial production as well as in the final product (4). New probiotics also include other microbes, such as yeast (e.g., *Saccharomyces boulardii*) and other quite different types of bacteria (e.g., *Clostridium butyricum*, *Bacillus subtilis*, etc.) (5, 6).

Lactobacilli are ubiquitous Gram-positive rod-like bacteria which have acid tolerance (pH 1-3) and beneficial antibacterial, antitumor, and antimutagenic effects (7, 8). The most consumed probiotic lactobacilli are *Lactobacillus acidophilus* and *Lactobacillus casei*. Lactobacilli have also been shown to inhibit the *in vitro* growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (9). This inhibition may be due to the production of organic acids such as lactic and acetic acid, hydrogen peroxide, bacteriocins, bacteriocin-like substances, and possibly biosurfactants (8). Recently, *Lactobacillus reuteri* has received considerable attention because of its beneficial effects on the health of the gastrointestinal tract (10) and its potential hypocholesterolemic effect (11). Also, this strain has been used in animal (piglet, chicken, turkey, etc.) and human (12).

Therefore, the objectives of this study are to identify and characterize the probiotic properties of the strain HY701 isolated from human feces for probiotic uses such as tolerance against gastric and bile acid, productivity of enzyme, and antimicrobial activity against *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* JM109.

Materials and Methods

Bacterial strain, culture media, and conditions Strain HY701 was isolated from human feces using lactobacilli MRS broth (Difco Lab., Detroit, MI, USA) at 37°C and was maintained at -70°C in MRS broth to which 20%(v/v) glycerol had been added. The culture was maintained in the laboratory with weekly transfer to MRS agar. The cells were grown in static culture at 37°C in MRS broth. *L. monocytogenes* ATCC 19111 and *E. coli* JM109 used for determination of the antimicrobial spectrum of activity were grown in TSA (Difco Lab.), Listeria selective agar (Unipath, UK) and EMB agar (BBL, Difco Lab.).

Identification of strain HY701 Strain HY701 was identified by Gram staining, morphology, and criteria based on carbohydrate fermentation patterns using an API 50 CHL kit (BioMeriux, Lyon, France), and 16S rDNA. 16S rDNA, chromosomal DNA extracted with a Wizard

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Genomic DNA purification kit (Promega, WI, USA) was analyzed by polymerase chain reaction (PCR) using universal primer (13). Amplification products were separated by electrophoresis and purified using a Wizard PCR Preps DNA purification system (Promega). The purified PCR product was inserted into topo vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

Artificial gastric acid tolerance Tolerance against gastric acid was measured by following the methods of Kobayashi (14, 15). Initially, cells were harvested by centrifugation at 3,860×g at 4°C for 10 min. *L. reuteri* HY701 was then suspended in MRS broth containing 1% pepsin, adjusted to pH 2.5 with 0.1 N HCl and cultured for 2 hr at 37°C. Viable cells were measured by incubating aliquots of these cultures on MRS agar plates for 24 hr at 37°C.

Artificial bile acid tolerance Artificial bile acid tolerance was determined by cultivating cells treated with artificial gastric acid at pH 2.5 containing 1% pepsin for 2 hr at 37°C. The cells were incubated in MRS broth containing 0.1% oxgall (Difco Lab.) for 0, 4, 8, 12, and 24 hr at 37°C. Viable cells were measured by incubating aliquots on MRS agar plates for 24 hr at 37°C.

Enzyme activity The API ZYM kit (BioMeriux) was used to study enzyme activity. *L. reuteri* HY701 was grown overnight at 37°C on MRS agar. Sediment from centrifuged broth culture was used to prepare a suspension of about 10^5 CFU/mL. After inoculation, broth was placed on the tray, covered with the plastic lid, and incubated for 4 hr at 37°C. By placing a surface-active agent (ZYM A reagent) in the cupules, solubilization of the ZYM B reagent in the medium was facilitated. The color was allowed to develop for at least 5 min. A value ranging from 0-5 was assigned, corresponding to the colors developed. The approximate number of free nmol of hydrolyzed substrate was determined by the color strength: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 or more nmol.

Antibiotic sensitivity Antibiotic sensitivity was determined by the paper disc method. Soft agar (0.75%), containing 10^7 cells of *L. reuteri* HY701 was overlaid on agar plates. After the medium solidified, a sterile paper disk was placed on the agar surface and 10 μL of antibiotic solution was immediately applied to each disk. Agar plates with antibiotic disks were then incubated at 37°C for 24 hr. The inhibition zone was then measured.

Antagonistic activity against *L. monocytogenes* ATCC 19111 and *E. coli* JM109 *L. monocytogenes* ATCC 19111 and *L. reuteri* HY701, or *E. coli* JM109 and *L. reuteri* HY701 were cultured for 12 hr, resuspended at 10^5-10^6 CFU/mL, and mixed in equal proportions (1:1) in a test tube (5 mL). Antagonistic activity was determined 0, 2, 4, 8, and 24 hr after incubation using the standard plate counting method. Viable cell numbers (CFU/mL) were determined on Listeria selective agar (Unipath) and EMB agar, respectively.

Results and Discussion

Identification of strain HY701 The strain HY701 was isolated from human feces by selecting isolates highly resistant to artificial gastric acid and bile acid. These tests showed strain HY701 to be a nonmotile, Gram-positive bacillus type (Table 1). Strain HY701 was identified as *Lactobacillus fermentum* with a confidence level of 96.9% using the API 50 CHL kit. Strain HY701 was also analyzed phylogenetically within the region occupied by the genus *Lactobacillus* using 16S rDNA sequences. Strain HY701 was identified as *L. reuteri* (Fig. 1). Therefore, strain HY701 was tentatively named *L. reuteri* HY701.

Artificial gastric acid and bile acid tolerance Incubation of *L. reuteri* HY701 at pH 2.5 containing 1% pepsin resulted in survival rates greater than 90% (Fig. 2). The initial cell number was 8.38 log CFU/mL, and the cell number remained at over 8 log CFU/mL after 1, 2, and 3 hr in artificial gastric acid. Most LAB grow more slowly at a low pH, and acid damage and loss of viability might

Table 1. Microbiological identification of strain HY701 by API 50 CHL kit[1)]

Carbon source	Strain HY701	Carbon source	Strain HY701
Control	-	Esculine	-
Glycerol	-	Salicine	-
Erythritol	-	Cellobiose	-
D-Arabinose	-	Maltose	+
L-Arabinose	+	Lactose	+
Ribose	+	Melibiose	+
D-Xylose	-	Saccharose	+
L-Xylose	-	Trehalose	-
Adonitol	-	Inulin	-
β-Methyl-xyloside	-	Melezitose	-
Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon	-
D-Fructose	-	Glycogen	-
D-Mannose	-	Xylitol	-
L-Sorbose	-	β-Gentiobiose	-
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	-	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-Acetylglucosamine	-	Gluconate	+
Amygdaline	-	2-Ketogluconate	-
Arbutine	-	5-Ketogluconate	-

[1)]+: utilized, -: not utilized.

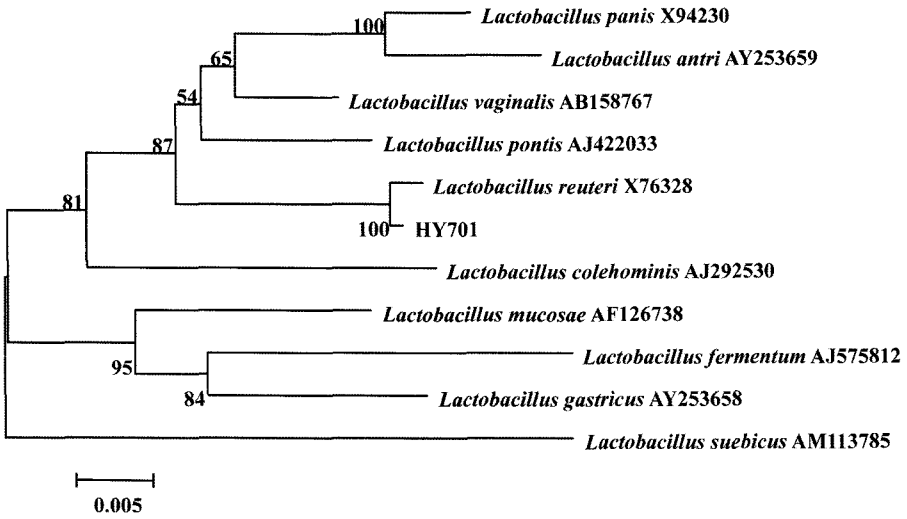

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the position of strain HY701 and representatives of some related taxa. Scale = 0.005 substitution per nucleotide position.

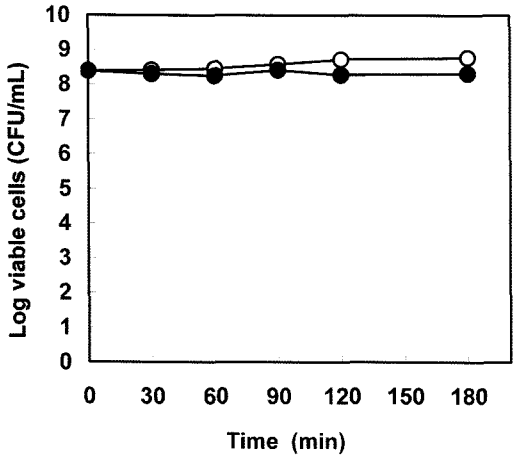

Fig. 2. Survival of *L. reuteri* HY701 in artificial gastric acid. –○–, non-treated with artificial gastric acid (control); –●–, treated with artificial gastric acid.

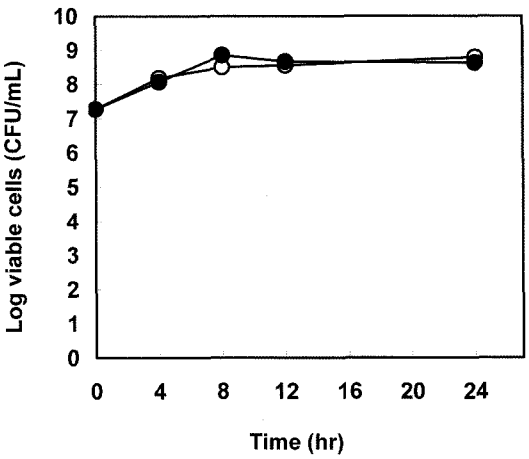

Fig. 3. Survival of *L. reuteri* HY701 in artificial bile acid after being treated with artificial gastric acid for 2 hr at 37°C. –○–, non-treated with artificial bile acid (control); – ● –, treated with artificial bile juice and bile acid.

also occur in cells held at low pH. However, *L. reuteri* HY701 remained stable at low pH for 2 hr. In similar studies, Mishra and Prasad (16) reported survival of *L. casei* strains at pH 1, 2, and 3. *L. acidophilus* strains tested exhibited growth at pH 3.0, and *L. bulgaricus* or *S. thermophilus* grew at low pH. *L. paracasei* and *L. rhamnosus* was almost unaffected by the low pH after 2 hr (17-20). *L. reuteri* CECT 925T was resistant to pH 3 for 1 hr (21).

One of the most important criteria in the selection of LAB for probiotic use is its resistance to bile salt, which is a prerequisite for the colonization and metabolic activity of these bacteria in the small intestine of the host (22). Cellular stress begins in the stomach, which has pH as low as 1.5 (23). After the bacteria pass through the stomach, they enter the upper intestinal tract where bile acid is secreted in the gut. The small intestine and colon of humans and animals contain relatively high concentrations of bile acids, which can inhibit growth or kill many bacteria. Therefore, it is essential that probiotic bacteria, to be effective, should be able to grow in 0.15-0.30% oxgall (18, 24). *L. reuteri* HY701 tested in this study was either resistant or tolerant to 24 hr of incubation in MRS broth supplemented with 0.1% oxgall. The results show that bile exerted a slight inhibitory effect in the growth of *L. reuteri* HY701 (Fig. 3). Its survival was lower than that of controls; however it was higher than the initiate (about 10^8 CFU/mL). It was thought the stagnant time of food in the stomach. It was able to accomplish for probiotic, if it got resistance to bile acid though low survival passed artificial gastric juice (25). *L. reuteri* CECT 925T was also resistant to 0.3% oxgall for 1 hr (21). From the above results, *L. reuteri* HY701 can likely survive passage through the gut and remain viable in the large intestine.

Enzyme production Enzyme production is one of the most important criteria in the selection of LAB for probiotic use. The enzymes trypsin, lipase, valine arylamidase,

Table 2. Enzyme activities of *L. reuteri* HY701 as determined by API ZYM kit

Enzyme	*L. reuteri* HY701[1)]
Control	0
Alkaline phosphatase	0
Esterase (C4)	3
Esterase lipase (C8)	2
Lipase (C14)	0
Leucine arylamidase	3
Valine arylamidase	0
Crystine arylamidase	0
Trypsin	0
α-Chymotrypsin	0
Acid phosphatase	4
Naphthol-AS-BI-phosphohydrolase	3
α-Galactosidase	4
β-Galactosidase	5
β-Glucuronidase	0
α-Glucosidase	0
β-Glucosidase	0
N-Acetyl-β-glucosamidase	0
α-Mannosidase	0
α-Fucosidase	0

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

crystine arylamidase, α-fucosidase, α-chymotrypsin, α-glucuronidase, β-glucuronidase, α-mannosidase, and N-acetyl-β-glucosamidase were not produced by *L. reuteri* HY701 (Table 2). However esterase (C4), acid phosphatase, esterase lipase (C8), α-chymotrypsin, naphthol-AS-BI-phosphahydroase, α-galactosidase, and β-galactosidase activities were produced. β-Glucuronidase is known to be a carcinogenic enzyme. When carcinogenic substances such as a benzo(a)pyrene come into the human body, its poisonous effects are neutralized by combination with glucuronic acid in liver. However, if it is excreted with bile juice in the intestine, this conjugate is cleaved by β-glucuronidase and it can become virulent again (26). The β-galactosidase activity of *L. reuteri* HY701 was not higher than that of *L. acidophilus* selected from Korean feces, but its activity was confirmed to be very strong (40 nmole) (2). β-Galactosidase is an essential enzyme for lactobacilli to utilize lactose and has been shown to reduce the symptoms of lactose intolerance in the animal or human body. Therefore, strain HY701 could alleviate lactose intolerance associated with functional foods including milk.

Antibiotic sensitivity The determination of antibiotic tolerance is necessary to select suitable cells for a patient taking antibiotics, and it clarifies this microbiological characteristic (27). Probiotics have some antibiotic tolerance, which does not change. Therefore safety is not connected with antibiotic tolerance. *L. reuteri* HY701 was sensitive to 100 μg/mL nisin, 20 μg/mL roxithromycin, and 15 μg/mL erythromycin, but showed tolerance to 20 μg/mL streptomycin, 10 μg/mL tetracycline, 20 μg/mL ciprofloxacin, 20 μg/mL nystatin, 20 μg/mL gentamycin, 10 μg/mL doxycycline, 10 μg/mL chloramphenicol, and 20 μg/mL ampicillin (Table 3).

Table 3. Antibiotic resistance of *L. reuteri* HY701

Antibiotics	Concentration (μg/mL)	*L. reuteri* HY701[1)]
Ampicillin	20	+
	30	+
Chloramphenicol	20	+
	30	+
Ciprofloxacin	10	+
	20	+
Doxycycline	20	+
	30	+
Erythromycin	15	-
	30	-
Gentamycin	10	+
	20	+
Nisin	50	+
	100	-
Nystatin	10	+
	20	+
Roxithromycin	20	-
	30	-
Streptomycin	10	+
	20	+
Tetracycline	10	+
	20	+

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

Antagonistic activity against *L. monocytogenes* ATCC 19111 and *E. coli* JM109 After an antibiotic is prescribed for a patient, probiotic strains are used to repress normalization of intestinal bacteria, as well as infection and growth of pathogenic bacteria in the intestine. *L. monocytogenes* is recognized as an important food-borne pathogen as a result of its wide distribution, and its ability to survive under adverse conditions such as extreme pH, heat, and cold is well known (28). As shown in Fig. 4, *L. reuteri* HY701 inhibited the growth of *L. monocytogenes* ATCC 19111 within 24 hr, therefore demonstrating strong bactericidal activity. *E. coli* JM109 growth was also inhibited by *L. reuteri* HY701 (Fig. 5). Some LABs produce bacteriocin-like substances which are proteinaceous and have antimicrobial activity (29-32). In particular, reuterin is a broad spectrum antimicrobial compound produced by some strains of *L. reuteri* (21). Also, some strains of *L. reuteri* (JCM1112, JCM1081, TM105, and NS128) were shown to inhibit binding of *Helicobacter pylori* to glycoplipid receptors (33). Since they may produce bacteriocin or other various antimicrobial substances, *L. reuteri* HY701 could effectively inhibit these pathogens in the intestines.

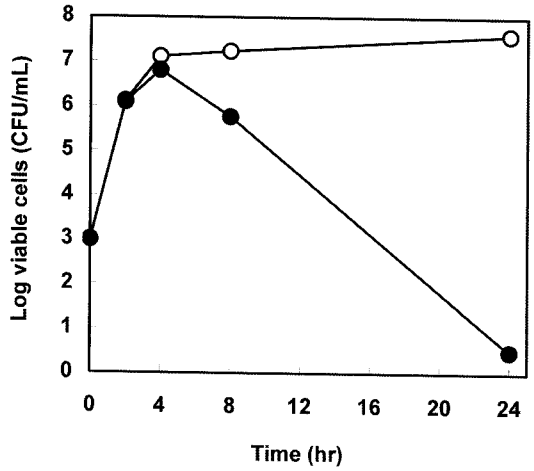

Fig. 4. Growth inhibition of *L. monocytogenes* ATCC 19111 by *L. reuteri* HY701. – ○ –, non-treated with *L. reuteri* HY701 (control); – ● –, treated with *L. reuteri* HY701.

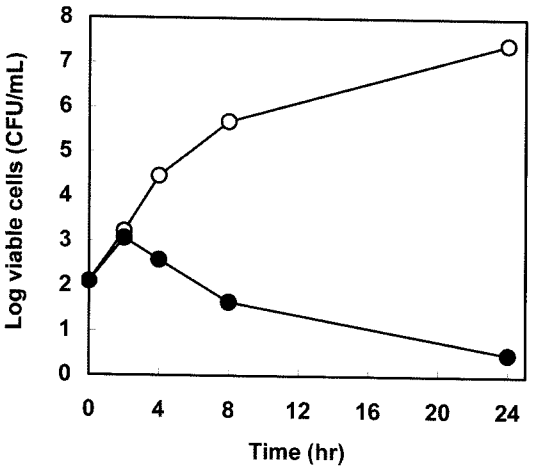

Fig. 5. Growth inhibition of *E. coli* JM109 by *L. reuteri* HY701. –○–, non-treated with *L. reuteri* HY701 (control); –●–, treated with *L. reuteri* HY701.

The results obtained indicate that *L. reuteri* HY701 has potential use in probiotic products. We conclude that *L. reuteri* HY701 possesses a number of interesting properties that constitute the basis for continued investigation of its use as a health-promoting substance.

Acknowledgments

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