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Selection of Human-Originated *Lactobacillus acidophilus* For Production of Probiotics

KIM, WANG-JUNE*, SEOK-SAN HONG AND SEONG-KWAN CHA

Korea Food Research Institute, Division of Food Biotechnology
San 46-1, Baekhyun-Dong, Bundang-Gu, Songnam-Si, Kyonggi-Do 463-420, Korea

Lactobacillus acidophilus KFRI 233, a strain isolated from human, was selected as a candidate for probiotics due to its excellent growth in MRS broth where no special anaerobic condition is required. Both simultaneous and deferred agar diffusion assays exhibited *Lb. acidophilus KFRI* 233 to possess an antagonistic effect against *Clostridium perfringens*. Its antagonistic effect was pH dependent. Associative culture of KFRI 233 and *Cl. perfringens* in broth resulted in maximum 94.04% inhibition of *Cl. perfringens*. β-Galactosidase activity of KFRI 233 was higher than other tested strains that are sold as commercial probiotics. Survival of KFRI 233 in pasteurized skim milk (4°C) and Sherbet mix (−15°C) after 7 days of storage were 71.9 and 105.5%, respectively.

Since the discovery of Metchnikoff, who stated that the daily consumption of lactic fermented milk is good for the health (12), there have been numerous researches conducted on the selection and development of suitable probiotic lactic acid bacteria for the enhancement of the human health. The beneficial effects of probiotics for human health has been reviewed thoroughly (3). Probiotics may be defined more fully as 'a live microbial feed supplement which beneficially affects the host animals by improving its intestinal microfloral balance' (3). Havenaar and Huis in't Veld (8) also described it as 'a mono or mixed culture of live microorganisms which, applied to man or animal (e.g. as dried cells or as fermented products), affects beneficially the host by improving the properties of the indigenous microflora'. The most widely used probiotic lactic acid bacteria are *Lb. acidophilus* and *Bifidobacterium* spp. (3).

There are many companies worldwide producing probiotics. However, there is only one in Korea. Annually, approximately fifty billion wons are spent on buying probiotic powder from abroad, and the amount is increasing. This led us to select an appropriate human-originated *Lb. acidophilus* strain which meet the requirements of good probiotics (3, 6). In this article, we report several physiological characteristics of the selected *Lb. acidophilus* KFRI 233.

*Corresponding author
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MATERIALS AND METHODS

Microorganisms, Media, and Culture Conditions

Human originated strains of *Lb. acidophilus*, designated as KFRI 217, 233, 491, 493, 507, were obtained from Korean Collection for Type Cultures, and the other *Lb. acidophilus* strains (KFRI 561, 572, 582), which were chosen from the commercial probiotic products sold in Korea, were kindly given by the College of Agriculture and Life Sciences, Seoul National University (Suwon, Korea). *Cl. perfringens* KFRI 434 was purchased from Korea National Institute of Health. Stock cultures of tested strains maintained in glycerol stock (−70°C) were thawed and transferred twice to a proper medium before use. Strains of *Lb. acidophilus* and *Cl. perfringens* were anaerobically grown in MRS broth (Difco) and Reinforced Clostridial Medium (RCM, Difco), respectively, at 37°C for 16 h. Hereafter, the anaerobic culture equipment used is GasPak Pouch™ (order # 60651, BBL) unless stated otherwise.

Growth of Strains Under Anaerobic and Oxygen Reduced Conditions

To select *Lb. acidophilus* strains that would grow well under the conditions where special anaerobic culture equipment is not required, exactly 1% inoculum of ovemight grown test strains were inoculated in MRS broth and grown (37°C, 16 h) under two different culture conditions: anaerobic and oxygen reduced (OR) conditions. To generate OR condition, the cap of the screw cap test tube (16×125 mm) containing 15 ml MRS

broth was immediately tightened after autoclaving, and the cell was incubated without GasPak Pouch. The colony forming unit (CFU)/m*l* in each condition was compared by surface plating and anaerobically incubating the cells on MRS agar supplemented with 0.05% cysteine hydrochloride (Sigma).

Inhibition of *Cl. perfringens* on Agar

Deferred and simultaneous triple agar diffusion method reported previously (9) was used to select strains that inhibit the growth of *Cl. perfringens*. The bottom and middle agar used were M (MRS agar), MC (MRS agar containing 0.05% cysteine hydrochloride) and MOC (MC supplemented with 0.15% oxgall). The top agar was RCM fortified with 0.7% agar (RCM soft agar). All plates were anaerobically incubated at 37°C for 24 h. The degree of antagonistic effect was expressed as S (smallest), M (medium), and L (large) by comparing the diameter of the inhibition zone.

Effect of pH on Antagonism

Selected strain that showed the largest zone of inhibition was further tested by agar well diffusion assay. Overnight grown cells in MRS broth under both the OR and anaerobic conditions were harvested by centrifugation (12,100×g, 10 min) and the supernatant fluid was divided into two parts: pH adjusted (pH 6.5) and unadjusted (pH 4.5). Both parts were filter sterilized (0.25 μm). Hundred μ*l* of each sample was loaded on the well (6 mm) that was aseptically made on Reinforced Clostridial Agar (RCA). After setting the temperature at 4°C for 1 h, 5 m*l* of RCM soft agar containing approximately 10^6 CFU of *Cl. perfringens* was poured and anaerobically incubated for 24 h. The presence or absence of inhibition zone was observed.

Inhibition of *Cl. perfringens* by Associative Culture

MRS broth supplemented with 0.5% sodium thioglycollate (MS) was chosen for the growth of both selected *Lb. acidophilus* strain and *Cl. perfringens* KFRI 434 (5). Two experiments were designed to create the population ratio in the inocula of *Lb. acidophilus* and *Cl. perfringens* KFRI 434 to be 1 : 1 and 10^4 : 1, in which the latter case simulates the ratio for both bacteria in human adult intestine. The broths were anaerobically incubated for 48 h at 37°C. As a control, the broth containing only *Cl. perfringens* KFRI 434 was used. The selective medium for the enumeration of *Cl. perfringens* KFRI 434 was Tryptose Sulfite Cycloserine (TSC) Agar which was prepared from individual ingredients. The % inhibition of *Cl. perfringens* KFRI 434 was obtained by the following equation:

$$\frac{(\text{CFU/m}l \text{ in control}) - (\text{CFU/m}l \text{ in associative})}{(\text{CFU/m}l \text{ in control})} \times 100$$

β-Galactosidase Activity

The β-galactosidase activities of the selected strain and commercial probiotic strains were compared. To induce the enzyme, cells were transferred twice in MGL broth (MRS broth containing 1% glucose and 0.5% lactose). Finally, cells were grown in 30 m*l* MRSL broth (MRS broth in which the glucose and beef extract were substituted by the same amount of lactose and tryptose, respectively) until they reached a late exponential phase of growth. Cells were then harvested by centrifugation (12,100×g, 5 min), washed twice with sterile distilled water and resuspended to its original volume in distilled water. Ten m*l* of cell suspension was transferred to a clean test tube. A cell-free extract was obtained by adding 0.5 m*l* a of solvent mixture containing acetone and toluene (9 : 1, v/v), vortexing at full speed for 20 sec, and was set at 37°C for 5 min. The standard reaction mixture consisted of 0.2 m*l* cell-free extract, 1.6 m*l* Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM 2-mercaptoethanol, pH 7.0, not autoclaved), and 0.2 m*l* of 10 mM o-nitophenyl-β-D-galactopyranoside (ONPG, Sigma). The mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 1 m*l* of ice cold 1 M Na_2CO_3. The absorbance (OD) was read at 420 nm and the amount of o-nitrophenol (ONP) liberated was determined from a standard curve (μM ONP vs. OD_{420}). The content of protein in the cell-free extract was determined by Coomassie Brilliant Blue binding microassay (2). A unit of an enzyme activity was defined as μM ONP/μg protein/min.

Survival in Sherbet Mix and Skim Milk

The survival of the selected strain in Sherbet mix and pasteurized (95°C, 5 min) 10% skim milk was investigated. Overnight grown strain in MRS broth was harvested by centrifugation (12,100×g, 5 min), washed twice with 0.1% peptone water, and was added to foods to make approximately 10^8 CFU/g or m*l*. The Sherbet mix was frozen at −15°C and skim milk was stored at 4°C. Both samples were stored for 1 week. The CFU/m*l* of before and after storage in each sample were determined and % survival was calculated.

RESULTS AND DISCUSSION

Our first strategy in our experiment was to select strains of human origin that have some aerotolerance, so that the management of cells would be easy and the strain would be colonized in human intestine, that have high activity in β-galactosidase, and that exhibit antagonistic effect against *Cl. perfringens* one of the major undesirable bacteria in human intestine (3). From the five strains of *Lb. acidophilus*, KFRI 233 showed

Table 1. Growth of *Lb. acidophilus* strains in MRS broth under anaerobic and oxygen reduced conditions.

Test strain No.	Origin	Mean CFU/ml under	
		Anaerobic[a]	Oxygen reduced[b]
233	human	5.9×10^8	5.8×10^8
217	human	7.5×10^7	2.3×10^7
507	human	1.9×10^6	6.1×10^4
491	human	1.1×10^6	4.1×10^4
493	human	1.7×10^7	2.6×10^6

[a] Using GasPak Pouch, [b] Cap was tightened after boiling.

Table 2. Comparison of antagonistic effect of *Lb. acidophilus* strains against *Cl. perfringens* KFRI 434 on different agar media.

Test strain No.	Assay methods					
	Simultaneous[a]			Deferred[b]		
	M[c]	MC[d]	MOC[e]	M	MC	MOC
217	++	+	++	+	++	++
233	+++	++	+++	+++	++	+++
491	+	+	+	+	+	+
493	−	−	−	+	+	+
507	+	−	−	+	+	+

[a] *Lb. acidophilus* and *Cl. perfringens* were grown simultaneously.
[b] *Lb. acidophilus* was grown overnight, then *Cl. perfringens* was grown.
[c] MRS agar, [d] M+0.05% cysteine-HCl, [e] MC+0.15% oxgall.
+++, ++, +, −: largest, medium, smallest, and no zone of inhibition, respectively.

Table 3. Effect of pH on the inhibition of cell-free supernatant fluid of KFRI 233 against *Cl. perfringens* KFRI 434.

pH value	Zone of inhibition
4.5[a]	absence
6.5[b]	presence

[a] pH of the overnight grown culture.
[b] pH of the overnight grown culture broth was adjusted by 3N NaOH.

the highest CFU/ml in both anaerobic and OR conditions, and there was no significant difference between the numbers in each condition (Table 1). Other strains showed comparable CFU/ml with KFRI 233 in anaerobic condition, however, their CFU/ml were remarkably lowered in OR condition.

The strain 233 also exhibited highest inhibitory effect against *Cl. perfringens* KFRI 434 by two agar diffusion methods (Table 2). Besides, its inhibitory effect was observed when it was anaerobically cultured on agar containing 0.15% oxgall in which the human intestinal condition was simulated. Some researchers have reported that bile resistant strains are better in colonization and growth in human intestine than bile sensitive ones (4, 6). This strongly indicates that strain 233 could behave as a good probiotic bacteria *in vivo*.

The inhibitory effect of cell-free supernatant fluid was

Table 4. Percent inhibition of *Cl. perfringens* KFRI 434 by associative culture.

	Mean CFU/ml in		% Inhibition	pH[a]
	Control	Associative		
Exp 1	5.5×10^8	1.2×10^8	78.2	4.56
Exp 2	1.73×10^8	1.03×10^7	94.04	4.57

[a] Final pH of sodium thioglycollate broth.
Exp 1: The population ratio in the inocula of *Lb. acidophilus* KFRI 233 and *Cl. perfringens* KFRI 434 was 1 : 1.
Exp 2: The population ratio in the inocula of *Lb. acidophilus* KFRI 233 and *Cl. perfringens* KFRI 434 was 10^4 : 1.

Table 5. β-Galactosidase activities of commercial strains and KFRI 233.

Test strain No.	μM ONP	μg protein/ml	Unit of activity[a]
572	10,000	2.1	481.1
582	2,600	1.1	236.3
233	7,600	2.01	378.1
561	2,500	3.1	80.6

[a] μM ONP/μg protein/min.

observed only from the one where the pH was adjusted to 6.5 (Table 3). This implies the inhibition was not caused by acid or H_2O_2. Here, we can carefully draw a speculation that the third inhibitory factor, *i.* e. bacteriocin, may be involved in the phenomenon. It is well documented that the activity of bacteriocin produced by many *Lb. acidophilus* strains is observed only when their pH was neutralized (10). Further studies, *i.* e. treatment of the supernatant fluid with several proteolytic enzymes, are necessary to confirm the involvement of bacteriocin.

Associative culture of strain 233 and *Cl. perfringens* resulted in maximum 94.04% inhibition of *Cl. perfringens* (Table 4). Comparable studies also have been reported (5). It is not clear, however, at this moment, what major factors are involved in inhibiting the growth of *Cl. perfringens* in broth. Populational dominance of strain 233 over *Cl. perfringens*, which leads to a depletion of nutrient and concomitant pH reduction, or the production of bacteriocin-like compound could be answer.

The lactose intolerance is a common problem for Mongolian (1). It seems reasonable that feeding the strain 233, that has a high β-galactosidase activity, as a dietary adjunct or a probiotic products could alleviate the problem. A comparison of the ability of the strains including KFRI 233 and commercial ones revealed considerable variations in their β-galactosidase activities (Table 5). Among examined strains, KFRI 233 showed a very high β-galactosidase activity. In comparison of the data made from other research (15), the β-galactosidase activity of strain 233 is remarkably high.

Table 6. Survival of strain 233 in Sherbet mix and skim milk after 7 days of storage[a].

Sample	Mean CFU/ml		% Survival	pH change
	Before storage	After storagen		
Sherbet mix	2.53×10^8	1.82×10^8	71.9	0.1
Skim milk	2.53×10^8	2.67×10^8	105.5	0.3

[a] Sherbet and skim milk were stored at −15° and 4°C, respectively.

The survival of strain 233 in Sherbet mix and skim milk after 7 days of storage was 71.9 and 105.5%, respectively (Table 6). Rasic and Kurmann claimed that 10^6~10^7 organisms per day would ensure the transit of viable bacteria through the stomach (14). Counts of strain 233 being always in the millions indicate that the strain 233 could survive in sufficient numbers to be useful as dietary adjunct. Similar results also stated *Lb. acidophilus* survived well in frozen yogurt (7, 11). With the increasing popularity of frozen dairy products, a huge market could be developed for dairy desserts containing live lactic acid bacteria. In this respect, strain 233 has several potentials.

In conclusion, strain 233 has several benefits: it is of human origin, easy to culture without expensive anaerobic equipment, inhibits the growth of *Cl. perfringens*, shows high β-galactosidase activity, resistant to bile, and maintains its high number in frozen food. Techniques such as formulation of less expensive medium, neutralization of pH, harvesting of cells, selection of cryoprotectant must be developed for the industrial production of probiotics using strain 233.

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