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ARTICLE

Probiotic Characteristics of *Lactobacillus plantarum* FH185 Isolated from Human Feces

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Abstract

Lactobacillus plantarum FH185 was isolated from the feces of healthy adults. In our previous study, *L. plantarum* FH185 was demonstrated that it has anti-obesity effect in the *in vitro* and *in vivo* test. In order to determine its potential for use as a probiotic, we investigated the physiological characteristics of *L. plantarum* FH185. The optimum growth temperature of *L. plantarum* FH185 was 40°C. *L. plantarum* FH185 showed higher sensitivity to novobiocin in a comparison of fifteen different antibiotics and showed higher resistance to polymyxin B and vancomycin. It also showed higher β-galactosidase and N-acetyl-β-glucosaminidase activities. Moreover, it was comparatively tolerant to bile juice and acid, and inhibited the growths of *Salmonella* Typhimurium and *Staphylococcus aureus* with rates of 44.76% and 53.88%, respectively. It also showed high adhesion activity to HT-29 cells compared to *L. rhamnosus* GG.

Keywords: *Lactobacillus plantarum*, probiotic characteristic, human feces

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Introduction

The word 'probiotics,' which is derived from the Greek and means *for life,* was first used by Lilley and Stillwell (1965) to describe the substances secreted by one microorganism to stimulate the growth of another, as an antonym of 'antibiotic'. In 1974, Parker defined probiotic "as organisms and substances which contribute to intestinal microbial balance". Fuller (1989) redefined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance".

Lactic acid bacteria (LAB) have complex nutritional requirements and are frequently used as probiotics or in the fermentation of food products. Probiotics consisting of one or more species of live bacteria, such as *Lactobacillus* and *Bifidobacteria*, not only affect the intestinal flora directly, but also affect other organs by modulating immunological parameters and intestinal permeability and producing bioactive or regulatory metabolites (de Vrese and Schrezenmeir, 2008; Delzenne *et al.,* 2011; Gerritsen *et al.,* 2011). Probiotics produce a health benefit when administered to animals, including humans. Several studies have reported the health-promoting effects of probiotics, including the maintenance of intestinal mucosal resistance to pathogenic microorganisms (Mennigen and Bruewer, 2009), prevention of diarrhea (Guandalini, 2008), stabilization of gut microflora (Gibson *et al.,* 1997), alleviation of lactose intolerance (de Vrese *et* al., 2001), immunomodulation (Perdigón *et al*., 2001), reduced serum cholesterol levels (Nguyen *et al*., 2007), reduction of bodyweight and metabolic disorders (Lee *et al*., 2007), and the prevention of allergic diseases and cancers (Isolauri and Salminen, 2008; Kumar *et al.*, 2010).

Probiotics must be safe for their intended use. The 2002 FAO/WHO guidelines recommend that, though bacteria may be generally recognized as safe (GRAS), the safety of a potential probiotic should be assessed by the minimum required testing, i.e., determination of antibiotic resistance patterns, assessment of certain metabolic activities, acid and bile salt tolerance, ability to adhere to the intestinal epithelium of the hosts, antagonistic activity against pathogenic bacteria, and assessment of the ability to maintain their viability during processing and storage (Lin *et al.*, 2006; Lonkar *et al*., 2005; Rial, 2000; Schlundt, 2002).

In our previous study, *L. plantarum* FH185 was observed to exhibit lipase inhibitory activity of 70.09±2.04% and to inhibit the adipocyte differentiation of 3T3-L1 cells (18.63±0.98%) at a concentration of 100 μg/mL. It

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was also demonstrated that the strain has an effect on the reduction of adipocyte size and gut microbial changes in diet-induced obese mice. Thus, this study was performed to investigate the physiological characteristics of *L. plantarum* FH185 in order to determine its potential as a starter for functional food products.

Materials and Methods

Bacterial strains

A LAB strain having an anti-obesity effect, namely, *L. plantarum* FH185, was isolated from the feces of healthy adults. In our previous study, *L. plantarum* FH185 was found to have lipase inhibitory activity of 70.09±2.04% and to inhibit the adipocyte differentiation of 3T3-L1 cells (18.63±0.98%) at a concentration of 100 μg/mL. It was also demonstrated that the strain has an effect on the reduction of adipocyte size and gut microbial changes in diet-induced obese mice (Park *et al*., 2015). The strain was incubated in a Lactobacilli MRS broth (Difco, USA) as the growth medium at 37°C for 18 h.

Growth of strain

The number of viable *L. plantarum* FH185 was determined by serial ten-fold dilution in 0.1% peptone water. Ten microliter of *L. plantarum* FH185 was inoculated into 150 mL of 10% reconstituted skimmed milk (10^5 CFU/mL), and then the culture was incubated at 3 h intervals for 24 h at 34°C, 37°C and 40°C. All of the pour plates were incubated aerobically at 37°C for 48 h using a BCP plate count agar (Eiken, Japan).

Antibiotic tolerance

L. plantarum FH185 was grown at 37°C for 18 h in MRS broth and inoculated (1%, v/v) into a MRS broth supplemented with antibiotics (amikacin, gentamicin, kanamycin, neomycin, streptomycin, penicillin-G, methicillin, oxacillin, ampicillin, bacitracin, rifampicin, novobiocin, lincomycin, polymyxin B and chloramphenicol; Sigma) at various concentrations in a two-fold dilution step. The minimal inhibitory concentration (MIC) was determined by checking the moment at which the strain stopped growing after incubation at 37°C for 48 h.

Enzyme activity

An API ZYM kit (Apibio-Mérieux) was used to study enzyme activity. *L. plantarum* FH185 was grown at 37°C for 18 h in MRS broth. Sediment from the centrifuged broth culture was used to prepare the suspension at 10^5-10^6 CFU/mL. After inoculation, the cultures were incubated for 5 h at 37°C. The addition of a surface active agent (ZYM A reagent) to the cupules facilitated the solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values ranging from 0-5 (corresponding to the colors developed) were assigned. The approximate number for the free nmol 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.

Bile tolerance

Bile tolerance was tested as described by Gilliland and Walker (1990). *L. plantarum* FH185 was grown at 37°C for 18 h in MRS broth. Each 1% of the *L. plantarum* FH185 strain culture was inoculated into sterilized MRS broth containing 0.05% L-cysteine (Sigma) with or without 0.3% oxgall (Sigma), and then the growth potential was compared in the presence of the bile. Then, the cultures were incubated anaerobically at 1 h intervals for 7 h at 37°C. All of the pour plates were incubated anaerobically at 37°C for 48 h using a BCP plate count agar.

Acid tolerance

Acid tolerance was tested as described by Clark *et al* (1993). Solutions of 37% HCl in double-distilled water were adjusted to pH levels of 2.0, 3.0, and 4.0. Sterile double-distilled water (pH 6.4) served as the control. 10 mL of each pH solution was transferred into sterile test tubes. One milliliter of stock culture containing approximately 10^9 CFU/mL of *L. plantarum* FH185 using MRS agar containing 0.05% cysteine was then transferred into each of the four pH solutions. The pH solutions containing *L. plantarum* FH185 were then incubated anaerobically at 37°C, followed by intermittent plating after 1, 2, and 3 h to stimulate the survival of *L. plantarum* FH185 under pH conditions common to the human stomach. Samples from the pH solution were re-suspended and subjected to serial dilutions. All of the pour plates were then incubated anaerobically at 37°C for 48 h using a BCP plate count agar.

Antimicrobial activity

Antimicrobial activity was tested as described by Gilliland and Speck (1977). *Escherichia coli* KFRI 174, *Salmonella* Typhimurium KFRI 250, and *Staphylococcus aureus* KFRI 219 (obtained from the culture collection of the Korea Food Research Institute [Korea]) were enumerated on an EMB agar (Difco), on a Bismuth sulfite agar

(Difco), and on a Baird Parker agar (Difco), respectively. All of the plates were incubated for 48 h at 37°C. Both the control culture and the associative culture were incubated for 6 h at 37°C. At the end of the incubation period, the samples were removed and placed in an ice bath until analysis. The number of CFU of pathogens per mL was determined using the appropriate selective medium. The percentages of inhibition were determined using the following formula:

$$\text{Inhibition (\%)} = \left[\frac{(\text{CFU/mL in control}) - (\text{CFU/mL in associative culture})}{(\text{CFU/mL in control})}\right] \times 100$$

Adherence assay

The adhesion of *L. plantarum* FH185 was studied using the HT-29 intestinal epithelial cell line (Kim *et al*., 2008). HT-29 cells were obtained from the Korea Cell Line Bank (Korea). The cells were cultured at 37°C in a 5% CO_2-95% air atmosphere in RPMI 1640 (GIBCO) supplemented with 10% FBS. The sub-cultured (3 times) *L. plantarum* FH185 was harvested by centrifugation at 12,000 rpm for 3 min, and then washed three times with PBS to remove any remaining MRS broth. The washed bacteria were then re-suspended in an RPMI 1640 medium to an optical density at 600 nm (OD 600) of 0.5 (approximately 10^7 CFU/mL). The re-suspended bacteria were appropriately diluted and plated on a BCP plate count agar. To investigate the adhesion activity, post-confluent HT-29 cells were washed twice with PBS. After washing, 1 mL of the bacteria in the RPMI 1640 medium was added to each well of the tissue-culture plate (12 wells), which was then incubated for 2 h. After incubation, the cells were washed five times with sterile PBS and harvested with a trypsin-EDTA (0.25% trypsin and 0.02% EDTA; GIBCO). It was appropriately diluted and plated on a BCP plate count agar to determine the number of viable cell-associated bacteria.

Statistical analysis

The results are expressed as the mean±standard deviation (SD). Statistical analysis was performed with a statistical analysis system (SAS, SAS Institute Inc., USA). The significance of the differences was analyzed by conducting a one-way analysis of variance (ANOVA) with Duncan's multiple range tests. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Growth of strain

As the result of incubation of *L. plantarum* FH185 in 10% reconstituted skimmed milk at 34°C, 37°C and 40°C for 24 h, the highest growth rate identified at 40°C. The pH value was also the lowest at 40°C. The optimum growth temperature of *L. plantarum* FH185 was found to be 40°C (Fig. 1).

Antibiotic tolerance

Several studies on the antibiotic sensitivity and resistance of dairy starter bacteria were conducted over a period of many years (Salminen *et al.*, 1998). Although some resistance appeared to be a strain, a specific pattern for classification has not emerged (Reinbold and Reddy, 1974).

Table 1 shows the tolerance of the *L. plantarum* FH185 strain to sixteen kinds of antibiotics. The results showed that *L. plantarum* FH185 showed itself to be more sensitive to novobiocin and penicillin-G in a comparison of fourteen different antibiotics, and exhibited the greatest resistance to polymyxin B and vancomycin.

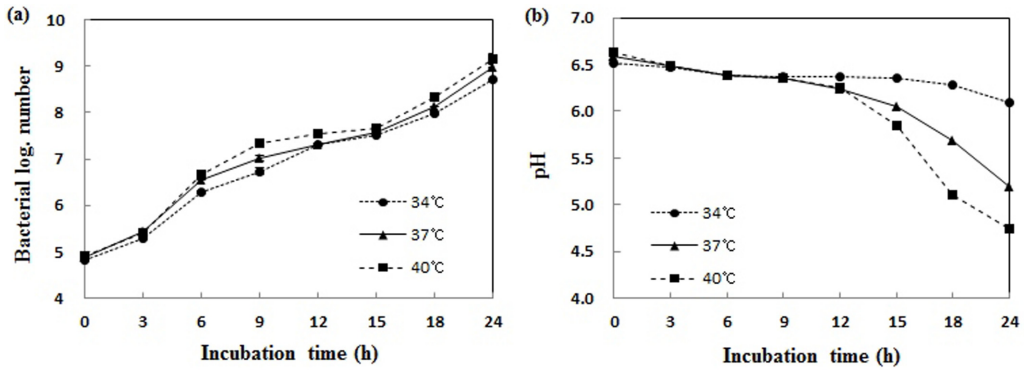

Fig. 1. Growth curve (a), and pH changes (b) of *Lactobacillus plantarum* FH185 in MRS broth at various temperatures. (a) All values are within the mean±standard deviation of the three replicates.

Table 1. Antibiotics susceptibility of *Lactobacillus plantarum* FH185

Antimicrobal agents	Minimal inhibitory concentrations (μg/mL)
Aminoglycosides	
Amikacin	20±0
Gentamycin	80±0
Kanamycin	100±0
Neomycin*	12.5±0
Streptomycin	400±0
β-lactams	
Penicillin-G*	10±0
Methicillin	160±0
Oxacillin	30±0
Ampicillin	320±0
Gram-positive spectrum	
Bacitracin*	15±0
Rifampicin	240±0
Novobiocin	7.5±0
Lincomycin*	12.5±0
Gram-negative spectrum	
Polymyxin B*	1200±0
Broad spectrum	
Chloramphenicol	80±0
Vancomycin	1600±0

*units/mL
All values are the mean±standard deviation of three replicates.

Table 2. Enzyme patterns of *Lactobacillus plantarum* FH185

Enzyme	*L. plantarum* FH185
Alkaline phosphatase	1
Esterase (C4)	1
Esterase Lipase (C8)	1
Lipase (C14)	1
Leucine arylamidase	4
Valine arylamidase	1
Cystinearylamidase	2
Trypsin	0
α-chymotrypsin	1
Acid phosphatase	3
Naphtol-AS-BI-phosphohydrolase	3
α-galactosidase	3
β-galactosidase	5
β-glucuronidase	0
α-glucosidase	3
β-glucosidase	4
N-acetyl-β-glucosaminidase	5
α-mannosidase	0
α-fucosidase	1

*A value ranging from 0 to 5 is assigned to the standard color: zero represents a negative; 5 represents a reaction of maximum intensity. Values 1 through 4 represent intermediate reactions depending on the level of intensity. The approximate activity may be estimated from the color strength: 1 corresponds to the liberation of 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles, and 5 to 40 nanomoles or more.

Vancomycin resistance is a matter of great importance in that vancomycin is one of the last antibiotics to remain widely efficacious against clinical infections caused by multidrug-resistant pathogens (Zhou *et al*., 2005). A few gram-positive bacteria, including *Lactobacillus* species, are essentially resistant to vancomycin (Swenson *et al*., 1990; Hamilton-Miller and Shah, 1998). Irreversible loss of antibiotic resistance from a strain as a result of a treatment known to eliminate plasmids is an indication that the resistance is plasmid-linked. However, in the case of the *Lactobacillus* strain, there has been no indication so far that vancomycin resistance would represent an inducible, transmissible genetic system (Salminen *et al*., 1998). For confirmation of the safety, cloning and expression of the gene related to anti-obesity effect from *L. plantarum* FH185 could be an alternative way.

Enzyme activity

There are many causes of cancer. The formation of carcinogens might be due to an association of bacterial enzymes like β-glucuronidase and nitroreductase, which are involved in the transformation of pro-carcinogens into carcinogen (Goldin, 1990). *L. plantarum* FH185 did not produce β-glucuronidase; rather, it produced such enzymes as leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosaminidase. Especially, *L. plantarum* FH185 produced more β-galactosidase and N-acetyl-β-glucosaminidase than other enzymes (Table 2). According to Zielinska *et al*. (2015), β-glucuronidase was not produced by any *Lactobacillus* isolated from traditional fermented cabbage and cucumber. However esterase, leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α and β-glucosidase, and N-acetyl-β-glucosaminidase were detected. This enzyme profile is similar to that of the *L. plantarum* FH185 strain.

Bile and acid tolerance

Tolerance to gastric juice and bile salts is a crucial factor in the selection of probiotic strains (Caggia *et al*., 2015). In order to ensure their beneficial effects after consumption, probiotics must be viable in the food and survive the gastrointestinal ecosystem with a pH ranging from 1.0 to 3.0 in the stomach and approximate bile salt concentrations of 0.3 in the small intestine (Mainville *et al*., 2005; Shah, 2007).

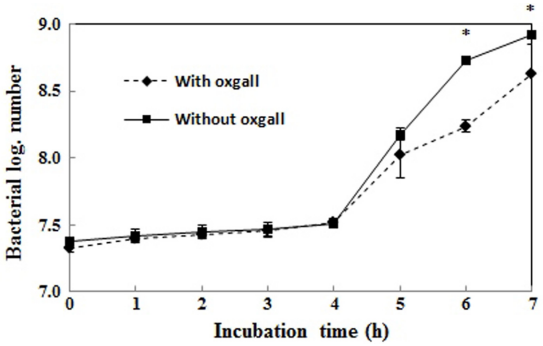

Fig. 2. Growth of *Lactobacillus plantarum* FH185 in MRS broth containing 0.05% L-cysteine with/without 0.3% ox gall. *$p<0.05$ between ox gall and without ox gall (*t*-test).

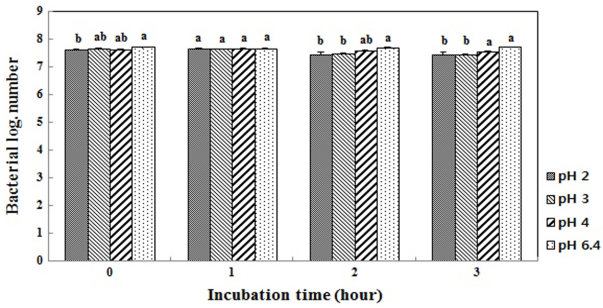

Fig. 3. Survival of *Lactobacillus plantarum* FH185 after three hours in HCl solution (pH 2.0, 3.0, 4.0 and 6.4). [a,b]Means values with different superscript within same time are significantly different ($p<0.05$).

Fig. 2 shows the growth curves in MRS broth or MRS broth containing 0.3% bile. The log value of the population after incubation for 7 h without 0.3% oxgall was 8.9, but it was 8.6 with the addition of 0.3% bile. Therefore, the survival rate of *L. plantarum* FH185 in MRS broth containing 0.3% bile was 96.6%. Also, Fig. 3 shows the pH tolerance of *L. plantarum* FH185. It showed a 97.4% survival rate after incubation for 3 h in highly acidic conditions (pH 2.0).

According to Guo *et al*. (2015), thirty kinds of *Lactobacillus* strains isolated from the suan-tsai and koumiss sample were tested with regard to their acid and bile tolerance. The acid resistance values of the lactobacilli ranged from 44.1 to 85.2%, while their bile tolerance values ranged from 4.6 to 34.2%. *L. plantarum* FH185 has probiotic potential because a comparatively high percentage of the strain survived in MRS broth containing 0.3% bile salt, under a highly acidic condition.

Antimicrobial activity

Foodborne diseases arising from the consumption of food contaminated with pathogenic bacteria such as *Salmonella* sp., *Listeria monocytogenes*, *Staphylococcus* sp., and *E.coli* is of vital concern to public health (Oussalah *et al.,* 2007). As the number of multidrug-resistant pathogens expands, and recognition of the role that human microbiota play in health and disease increases, it is becoming increasingly interesting to use probiotics as a therapeutic agent (Britton and Versalovic, 2008). For instance, many researchers have demonstrated the anti-pathogenic effects of LAB (Casey *et al.*, 2007). The antagonistic activities demonstrated by lactic acid bacteria may be due to the production of substances with antibacterial properties in particular: hydrogen peroxide, organic acid and bacteriocins (Tejero-Sarinena *et al*., 2012).

Table 3 shows the antimicrobial activity of *L. plantarum* FH185 against various pathogenic strains. *L. plantarum* FH185 did not show any inhibition against *E. coli*, but it showed inhibition against *S.* Typhimurium and *S. aureus* at rates of 44.4% and 53.9%, respectively. The pH value of pathogens after incubation for 7 h was 6.4, but the pH value of a mixed culture with *L. plantarum* FH185 and pathogens was around 5.5-5.6. This means that even lactic acid produced during incubation affected the antimicrobial activity, it was not a large effect. Although *L. plantarum* FH185 did not show resistance against *E. coli*, the strain showed comparatively excellent inhibition against *S.* Typhimurium and *S. aureus*.

Adhesion ability

Adherence ability to the intestinal epithelium of the

Table 3. Inhibition of pathogens by *Lactobacillus plantarum* FH185 in MRS broth

Indicators	Indicators[a]		*L. plantarum* FH185[a] + Indicators		Inhibition (%)
	CFU/mL	pH	CFU/mL	pH	
Escherichia coli	$2.0\pm0.2\times10^7$	6.4	$3.2\pm0.3\times10^7$	5.6	-
Salmonella Typhimurium	$7.2\pm0.4\times10^6$	6.4	$4.0\pm0.2\times10^6$	5.5	44.4
Staphyloccous aureus	$3.3\pm0.1\times10^8$	6.4	$1.7\pm0.3\times10^8$	5.6	53.9

*Initial count of *L. plantrum* FH185: $4.4\pm0.1\times10^6$ CFU/mL
[a]Determined after 6 h of incubation at 37°C
All values are the mean±standard deviation of three replicates.

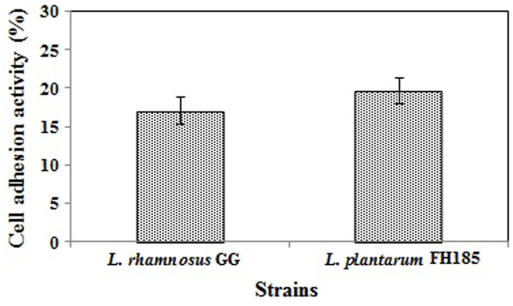

Fig. 4. Adhesive ability of *Lactobacillus plantarum* FH185 to HT-29 cell. All values are the mean±SD of three replicates. There are no significantly different (p<0.05).

hosts is one of the main criteria for selecting probiotic strains. Attachment to mucosa prolongs the time probiotics can influence the gastrointestinal immune system and microbiota of the host. Thus, the ability to adhere to intestinal surfaces is thought to correspond to the efficacy of the probiotic strain (O'Halloran *et al.*, 1997). As shown in Fig. 4, 19.62% of *L. plantarum* FH185 adhered to HT-29 cell and 17.02% of the *L. rhamnosus* GG strain adhered to the cell. *L. rhamnosus* GG was used as the positive control. In many studies, it was demonstrated that *L. rhamnosus* GG has a great ability to adhere to the epithelial cell line (Gopal *et* al., 2001; Tuomola and Salminen, 1998). Thus, we could define that *L. plantarum* FH185 exhibits great adherence to the epithelial surface.

Conclusion

In our previous study, *Lactobacillus plantarum* FH185 was isolated from the feces of healthy adults and demonstrated to have anti-obesity effects. We investigated the physiological characteristics of *L. plantarum* FH185 for potential use as probiotics. The essential and fundamental properties of probiotics - such as growth pattern, antibiotic tolerance, enzyme activity, bile tolerance, acid tolerance, antimicrobial activity and adhesion ability - were tested. The optimum growth temperature of *L. plantarum* FH185 was 40°C. *L. plantarum* FH185 was able to survive in antibiotic conditions at a low concentration and did not produce carcinogenic enzymes such as β-glucuronidase. Moreover, it was found to be comparatively tolerant to bile juice and acid, and displayed inhibition against two kinds of pathogenic strains. It also showed high adhesion activity to HT-29 cells compared to *L. rhamnosus* GG. These results demonstrate that *L. plantarum* FH185 could be used as a probiotic.

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