

## Screening of Lactobacilli Derived from Chicken Feces and Partial Characterization of *Lactobacillus acidophilus* A12 as Animal Probiotics

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This study was performed to screen and select *Lactobacillus* strains from chicken feces for probiotic use in animals. Of these strains, strain A12 had the highest immunostimulatory effect. Therefore, strain A12 was characterized as a potential probiotic. Strain A12 was tentatively identified as *Lactobacillus acidophilus* A12, using the API 50 CHL kit based on a 99.9% homology. *L. acidophilus* A12 was highly resistant to artificial gastric juice (pH 2.5) and bile acid (oxgall). Based on results from the API ZYM kit, leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, and *N*-acetyl- $\beta$ -glucosamidase were produced by strain A12. *L. acidophilus* A12 showed resistance to several antibiotics (nisin, gentamicin, and erythromycin). The amount of interleukin (IL)-1 $\alpha$  in 20 $\times$  concentrated supernatant from *L. acidophilus* A12 was approximately 156 pg/ml. With regard to antioxidant activity, *L. acidophilus* A12 supernatant showed 60.6% DPPH radical scavenging activity. These results demonstrate the potential use of *L. acidophilus* A12 as health-promoting probiotics.

**Keywords:** *Lactobacillus acidophilus*, chicken feces, animal probiotics, interleukin-1 $\alpha$

Antibiotics have been used as feed additives to promote more productive livestock farming [23]. Growth-promoting antibiotics have been used therapeutically in animal feed to improve the health and well-being of animals and for prophylactic purposes to improve poultry production. However, the use of these growth-promoting substances has led to an

imbalance of beneficial intestinal flora and the development of antibiotic-resistant bacteria that are pathogenic to animals or humans [1, 27].

Probiotics have been used as an alternative to the use of antibiotics in animals and humans, and their efficiency as such in animals has been widely discussed [3, 9, 11, 24, 25, 32]. Probiotics are viable single or mixed cultures of microorganisms that when given to animals or humans, beneficially affect the host by improving the properties of the indigenous microflora. The bacteria most commonly associated with probiotic activity are lactobacilli and bifidobacteria, but nonpathogenic organisms, such as *Escherichia coli* and the yeast *Saccharomyces boulardii*, have also been used. Probiotic characteristics have been examined with regard to survival in gastric conditions, colonization of the intestine, reduction of lactose intolerance, prevention of antibiotic-induced diarrhea, cholesterol lowering effect, prevention of colon cancer, and stimulation of the immune system [12, 14, 22, 26, 28, 31, 34, 36].

Lactic acid bacteria are GRAS (Generally Recognized as Safe) organisms, and probiotics consisting of lactic acid bacteria primarily use *L. acidophilus*, *L. casei*, *L. reuteri*, and so on [4]. Many lactic acid bacteria produce antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, carbon dioxide, and bacteriocins [17]. These antimicrobials are used in humans and animals as probiotics for beneficial effects.

In this study, *Lactobacillus* spp. were isolated from chicken feces for probiotic use as an alternative to the use of antibiotics in animals. We then selected useful probiotic strains having an immunostimulatory effect. Among these strains, selected *Lactobacillus* spp. were investigated for probiotic characteristics such as tolerance to artificial gastric juice and artificial bile acid, enzyme production, antibiotic resistance, and so on.

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## MATERIALS AND METHODS

### Bacterial Strains and Culture Media

For the isolation of *Lactobacillus* spp. from chicken feces, 1 g of freshly collected feces from chickens was suspended (1:10 dilution) and incubated in 1% peptone water (pH 3.5) for 2 h at 37°C. One hundred  $\mu$ l of suitable dilutions were then spread onto plates containing LBS medium (*Lactobacillus* selective medium; BBL, Cockeysville, MD, U.S.A.). Isolates were incubated in lactobacilli MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) at 37°C, and stored as stock solutions in 20% (v/v) glycerol at -70°C.

### Production of Interleukin-1 $\alpha$ (IL-1 $\alpha$ )

Isolated *Lactobacillus* strain supernatants were concentrated by ethanol precipitation. IL-1 $\alpha$  in 20 $\times$  concentrated supernatants was measured by quantitative ELISA (enzyme-linked immunosorbent assay) using commercially available kits (Koma Biotech Inc., Seoul, Korea). The color reaction was measured at 450 nm with an ELISA plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

### Identification of Strain A12

Cell morphology, Gram-staining, and API 50 CHL medium were analyzed for the identification of strain A12. API 50 CHL medium (bioMerieux, Lyon, France) was used to study the carbohydrate use of the strain.

### Tolerance to Artificial Gastric Juice and Artificial Bile Acid

Analysis of artificial digestive fluid tolerance followed the method of Kobayashi *et al.* [18]. Initially, cells were harvested by centrifugation at 4,000  $\times$ g for 10 min at 4°C. *L. acidophilus* A12 was then suspended in MRS broth containing 1% (w/v) pepsin, adjusted to pH 2.5 with 0.1 M HCl, and cultured for 2 h at 37°C. Tolerance of artificial bile acid was determined by cultivating cells treated with artificial gastric juice. The cells were incubated for 24 h at 37°C in artificial bile acid consisting of MRS broth containing 0.1% (w/v) oxgall (Difco). Viable cells were measured by incubating aliquots on MRS agar plates for 24 h at 37°C.

### Analysis of Enzyme Activity

The API ZYM kit (bioMerieux) was used to study enzyme activity production by *L. acidophilus* A12. *L. acidophilus* A12 was grown overnight at 37°C on MRS broth. Sediment from centrifuged culture broth was used to prepare a suspension at 10<sup>5</sup> CFU/ml. After inoculation, cultures were incubated for 4 h at 37°C. Placing a surface-active agent (ZYM A reagent) in the cupules facilitated 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 were assigned corresponding to the colors developed. The approximate number of free nmol of 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.

### Antibiotic Sensitivity

Antibiotic sensitivity was determined by the paper disk method. Soft agar (0.75%, w/v), containing 10<sup>7</sup> cells of *L. acidophilus* A12, was overlaid on agar plates. After the medium had solidified, a sterile paper disk, which had been previously treated with antibiotics diluents, was laid on the agar and incubated at 37°C for 24 h. The zone of inhibition was measured from the edge of the disk.

### Scavenging Effect on DPPH Radicals (Electron Donating Activity)

The antioxidant activity of *L. acidophilus* A12 supernatant was assessed by measuring its ability to scavenge the stable DPPH (1,1-diphenyl-2-picrylhydrazyl; Sigma, U.S.A.) free radical. One ml of 100  $\mu$ M DPPH ethanol solution was added to 200  $\mu$ l of sample solution and allowed to react at room temperature. After 10 min, the absorbance values were measured at 528 nm using a spectrophotometer and converted into percentage antioxidant activity.

## RESULTS AND DISCUSSION

### Screening of *Lactobacillus* spp. from Chicken Feces

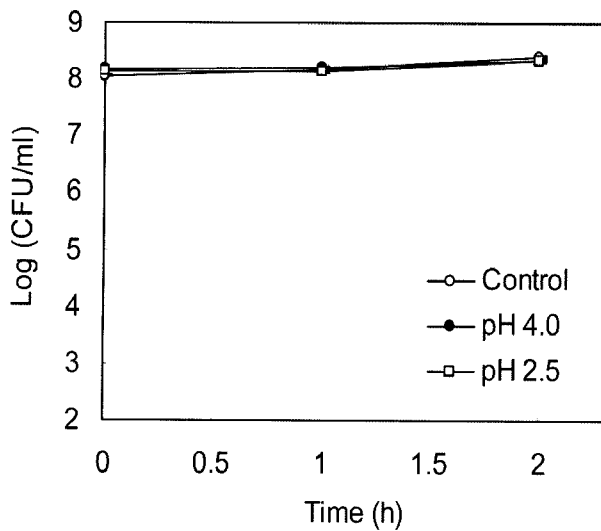
The study of lactic acid bacteria for their potential use as probiotics in farm animals is increasing. Current probiotics require the characteristics of stability in intestinal conditions, antagonistic effect, prevention of colon cancer, and immunostimulatory effects. Seven *Lactobacillus* spp. isolates from chicken feces were screened for tolerance to pH (pH 3.5 for 2 h). The immunostimulatory effect of probiotics is an increasingly important characteristic [6]. These isolates were then screened for immunostimulatory activity through IL-1 $\alpha$  production. The 20 $\times$  concentrated supernatants of B12, B23, B32, C23, and C31 strains did not show evidence of IL-1 $\alpha$  production, whereas 20 $\times$  concentrated supernatants of A12 and B31 were shown to have 156 and 33 pg/ml of IL-1 $\alpha$  production, respectively. In these *Lactobacillus* spp, strain A12 was selected for further analysis because of its level of IL-1 $\alpha$  production.

### Identification of Strain A12 as a Probiotic Strain

*Lactobacillus* strain A12 was identified using Gram-staining and its use of various carbon sources (data not shown). Strain A12 was a Gram-positive *Bacillus* strain. A tentative identification using the API 50 CHL kit showed strain A12 to have 99.9% similarity to *Lactobacillus acidophilus*. Thus, this strain was tentatively named as *L. acidophilus* A12.

### Tolerance to Artificial Gastric Juice and Artificial Bile Acid

Probiotic bacteria must survive the harsh environments in the stomach (low pH) and intestinal tract, which contains bile acid. *L. acidophilus* DC601, *L. bulgaricus*, *L. rhamnosus* DC427, and *Streptococcus thermophilus* were able to grow well at low pH [13, 36]. However, some lactic acid starter bacteria, such as *L. acidophilus* A3, A9, 08, 53, 5, CSL, CNRZ 1881, and CNRZ 1923, were reported to have losses in cell viability equal to 3.4–5.0 log of viable cells in gastric solutions of pH 2 and pH 3 [35]. Our results show that *L. acidophilus* A12 has a high survival rate of over 8 log of viable cells in artificial gastric acid (Fig. 1). In bile acid, *L. acidophilus* strains grew better than *L. casei*, *L. rhamnosus*, and bifidobacteria [20, 29, 35]. Similarly, at pH 2.5 and 4.0, over 99% of *L. acidophilus* A12 survived and there



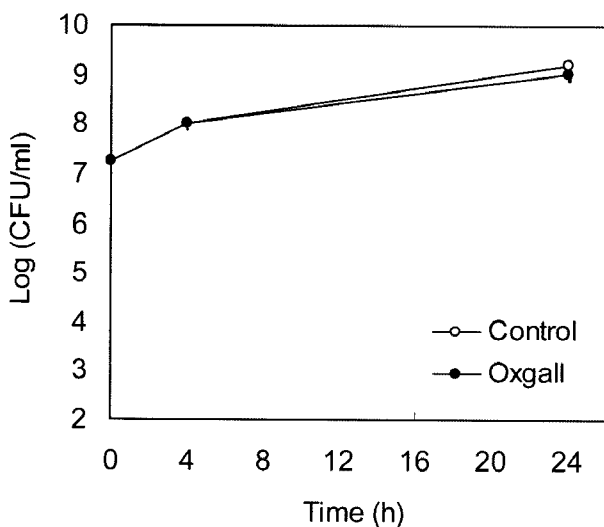
**Fig. 1.** Tolerance of *L. acidophilus* A12 to artificial gastric juice.

○, Nontreated (control, pH 7.0); ●, treated with artificial gastric juice (pH 4.0); □, treated with artificial gastric juice (pH 2.5).

was a 98.1% survival rate after 24 h incubation in medium with artificial bile acid (Fig. 2).

#### Enzyme Production by *L. acidophilus* A12

The enzyme production of *L. acidophilus* A12 was an important criterion in its selection, because carcinogenic enzymes such as  $\beta$ -glucuronidase can be produced by microorganisms [7]. When carcinogenic substances such as benzo(a)pyrene enter the human body, their poisonous effects are counteracted because of conjugation with glucuronic acid in the liver. If this conjugated product is excreted with bile acid



**Fig. 2.** Tolerance of *L. acidophilus* A12 to artificial bile acid after treatment with artificial gastric juice (pH 2.5) for 2 h at 37°C.

○, Nontreated (control); ●, treated with bile acid in artificial gastric juice.

**Table 1.** Enzyme activity of *L. acidophilus* A12 using the API ZYM kit.

Enzyme	<i>L. acidophilus</i> A12
Control	0
Alkaline phosphatase	0
Esterase	0
Esterase lipase	0
Lipase	0
Leucine arylamidase	4
Valine arylamidase	0
Cystine arylamidase	3
Trypsin	0
$\alpha$ -Chymotrypsin	0
Acid phosphatase	3
Naphthol-AS-BI-phosphohydrolase	5
$\alpha$ -Galactosidase	2
$\beta$ -Galactosidase	5
$\beta$ -Glucuronidase	0
$\alpha$ -Glucosidase	2
$\beta$ -Glucosidase	4
<i>N</i> -Acetyl- $\beta$ -glucosaminidase	4
$\alpha$ -Mannosidase	0
$\alpha$ -Fucosidase	0

0, 0 nmol; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5,  $\geq 40$  nmol.

in the intestine, cleavage by  $\beta$ -glucuronidase can liberate these substances to become toxic once again. *L. acidophilus* A12 did not produce the carcinogenic enzyme,  $\beta$ -glucuronidase, whereas  $\beta$ -galactosidase was produced, which is beneficial for lactose intolerance. *L. acidophilus* A12 produced enzymes including leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, and *N*-acetyl- $\beta$ -glucosaminidase (Table 1).

#### Antibiotic Sensitivity

Antibiotic sensitivity is important since many antimicrobial chemicals are used as feed additives. That is, probiotics need to have some antibiotic tolerance that does not change [37]. *L. acidophilus* A12 was shown to have broad resistance to nisin, gentamycin, and erythromycin, and  $< 200$   $\mu$ g/ml of streptomycin, roxithromycin, and chloramphenicol (Table 2). In contrast, medium containing neomycin or rifampicin showed growth inhibition of *L. acidophilus* A12. Similarly, *L. delbrueckii* subsp. *lactis* UO 004 is resistant to chloramphenicol and erythromycin [10].

#### Antioxidant Activity

Antioxidant activity was reported for synthetic (BHA and BHT) and natural (quercetin, kampferol, mycetin, vitamins C and E, etc.) antioxidants [33]. These compounds are found in many plant raw materials, particularly in fruits, seeds, and herbs [8, 15, 19, 30], and are produced during fermentation by microorganisms [2, 5, 21]. In *L. acidophilus* A12

**Table 2.** Resistance of *L. acidophilus* A12 to various antibiotics.

Antibiotics ( $\mu\text{g}/\text{m}^2$ )	<i>L. acidophilus</i> A12	
Nisin	2.5	+ <sup>a</sup>
	25	+
	50	+
	100	+
Streptomycin	5	+
	10	+
	20	+
	200	-
Neomycin	5	-
	10	-
	20	-
	200	-
Roxithromycin	5	+
	10	+
	20	+
	200	-
Chloramphenicol	5	+
	10	+
	20	+
	200	-
Gentamicin	5	+
	10	+
	20	+
	200	+
Rifampicin	5	-
	10	-
	20	-
	200	-
Erythromycin	5	+
	10	+
	20	+
	200	+

+, Growth; -, no growth.

supernatant, 60.6% DPPH radical scavenging activity was produced compared with *B. polyfermenticus* SCD, which was shown to have 56.5% DPPH radical scavenging activity [16].

This study examined the probiotic properties of bacterial strains from chicken feces. We showed that *L. acidophilus* A12 has a number of interesting probiotic properties, such as high survival in artificial gastric and bile acids, production of beneficial enzymes and interleukin (IL)-1 $\alpha$ , tolerance of antibiotics, and antioxidant production. These results demonstrate the potential use of *L. acidophilus* A12 as a health-promoting probiotic.

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