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ARTICLE

Screening of Immunostimulatory Probiotic Lactic Acid Bacteria from Chicken Feces as Animal Probiotics

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Abstract

The principal objective of this study was to screen and select acid-tolerant *Lactobacillus* strains from chicken feces, feeds, and other sources. Fourty six strains evidencing acid tolerance (pH 3.5) were isolated in this study. Among them, nine strains exhibited marked immunostimulatory effects. Therefore, nine candidate strains were characterized for probiotic use. In order to evaluate macrophage activation, NO production was measured using RAW 264.7 cells. In particular, three strains (FC812, FC222, and FC113) evidenced the highest levels of NO production measured at 38.39±20.01, 35.06±27.73, and 33.88±15.99 μM, respectively, at a concentration of 10^8 CFU/mL. The majority of strains, with the exception of strain FC322, evidenced marked resistance to artificial gastric juice (pH 2.5 with 1%(w/v) pepsin). Additionally, strains FC222, FC421, FC511, and FC721 were highly resistant to artificial bile acid (0.1%(w/v) oxgall), whereas strains FC113, FC322, FC422, FC621, and FC812 were the least resistant to bile. All nine strains exerted antimicrobial effects against chicken-related pathogens. Additionally, all nine strains were found to be resistant to several antibiotics. The isolated strains, except for strain FC322, were tentatively identified as *Lactobacillus salivarius*, using an API 50 CHL kit. These results demonstrate that some probiotic organisms may potentially probiotic properties, and thus may serve as an effective alternative to antibiotics in animal applications.

Key words: animal probiotics, immunostimulatory effect, chicken feces, lactic acid bacteria

Introduction

Antibiotics have been used as feed additives to promote the productivity of livestock farming, in addition to their conventional use in therapy (Wierup, 2001). However, the extensive use of antibiotics to promote animal growth rates has resulted in an imbalance in beneficial intestinal flora, as well as the appearance of resistant bacteria. Additionally, the presence of residual antibiotics in meat and eggs is unacceptable. In order to safeguard human health, the Food and Agriculture Organization/World Health Organization (FAO/WHO) have established standards for maximum residue limits (Lee and Choi, 2006; Muriuki *et al*., 2001; Pascual *et al*., 1999). Therefore, there is an increasing interest in probiotics as an alternative to the use of antibiotics.

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Probiotics have been defined by the FAO/WHO as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (Shanahan, 2004). The most common probiotic bacteria are the lactic acid bacteria, such as the lactobacilli and bifidobacteria, certain spore-forming *Bacillus* species, or yeasts like *Saccharomyces boulardii* (Hoa *et al*., 2000; Jun *et al*., 2000; Shin *et al*., 1999). Extensive studies have been conducted to determine the relevant characteristics of probiotics, including survival under gastric conditions, improvements in unbalanced intestinal microbiota, reductions of lactose intolerance, the prevention of antibiotic-induced diarrhea, reduction of cholesterol levels, prevention of colon cancer, and stimulation of immune system (De Rodas *et al*., 1996; Kimura *et al*., 1997; Pool-Zobel *et al*., 1996; Reddy *et al*., 1998; Sanders, 2003).

The lactic acid bacteria (LAB) are closely associated with human and animal environments. The LABs are classified as GRAS (Generally Recognized as Safe) organisms, and can be detected in the gastro-intestinal tracts of humans and animals, and also in fermented food (Holza-

pfel *et al.*, 1993; Klein, 2003). Various strains of LAB isolated from the gut evidence probiotic properties, as do fermented dairy products and vegetables (Vizoso Pinto *et al.*, 2006). They generate antimicrobial substances against undesirable pathogens including organic acid, hydrogen peroxide, ethanol, diacetyl, carbon dioxide, and bacteriocins (Ayad *et al.*, 2002; Caplice and Fitzgerald, 1999).

Nitric oxide (NO) performs a central role in several physiological functions, and is also operant in the onset and maintenance of certain pathological conditions, including immune system issues (Marin and Rodriguez-Martinez, 1997). NO interacts with cytokines or microbial compounds (Bogdan, 2001) and is generated during the oxidation of L-arginine to L-citrulline by the enzyme NO synthase (NOS) (Liao *et al*., 1995). Three isoforms of NOS are currently known: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Kanno *et al*., 2006). Among them, iNOS can generate high levels of NO in immune responses (Guzik *et al*., 2003), and the NO produced by iNOS is observed principally in cells of the macrophage-monocyte lineage such as monocytes and macrophages (Oleszak *et al*., 1997). In general, moderately high levels of NO may exert cytostatic or cytotoxic effects, including anti-bacterial, anti- viral, anti-protozoa, and anti-apoptotic effects on immune cells (Karpuzoglu and Ahmed, 2006; McCartney-Francis *et al*., 1993), whereas excessively high levels of NO are involved in inflammatory autoimmune diseases (Bogdan *et al*., 2000). Therefore, NO can be viewed as a highly bioactive, but potentially toxic molecule (Weisz *et al*., 1996).

In this study, we screened and selected *Lactobacillus* spp. from chicken feces for probiotic use, as an alternative to the use of antibiotics in animals. The selected strains were assessed with regard to their probiotic characteristics, including their resistances to artificial gastric juice and bile acid, antimicrobial activity, antibiotic susceptibility, and immunostimulatory effects.

Materials and Methods

Isolation of LAB

Samples of chicken feces were provided by Ahnil Farm in Yunchun, Korea. For the isolation of *Lactobacillus* spp. from chicken feces, 3 g of collected samples were suspended in 30 mL of 0.85% saline (pH 3.5) and incubated at room temperature for 2 h. One hundred μL of diluted suspensions were spread onto LBS (Lactobacillus selection medium; BD BBL, Cockeysville, MD, USA) agar plates. After the plates were incubated at 37°C for 24 h, colonies with different morphologies were selected. The isolates were grown in lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) at 37°C, and stored as stock solutions in 20% (v/v) glycerol at -70°C.

Identification of strains

The selected LAB strains were identified by their biochemical carbohydrate fermentation patterns, using an API 50 CHL kit (BioMerieux, Lyon, France). The colonies were initially suspended in API 50 CHL medium and the 50 compartments of the strips were inoculated. Incubation was conducted at 37°C under aerobic conditions, and the reactions were observed at 24 and 48 h. The biochemical profiles were obtained and entered into the identification program.

Cell culture

The murine macrophage cell line (RAW 264.7, KCLB 40071) was purchased from the Korean Cell Line Bank (KCLB; Seoul National University, Seoul, Korea). The cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium; Gibco Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco Laboratories) and 1% streptomycin-penicillin (Gibco Laboratories) at 37°C in an atmosphere of 5% CO_2/95% air. For the NO assay, the cells were seeded in new dishes and grown to 80% confluence.

Nitric oxide assay

The RAW 264.7 cells were seeded at a density of 2×10^4 cells/well in 96-well culture plates and incubated at 37°C for 24 h in an atmosphere of 5% CO_2/95% air. RAW 264.7 cells were activated via the addition of heat-treated (100°C, 30 min) suspension of the LAB stains selected in medium at concentrations of 1×10^6, 1×10^7, and 1×10^8 CFU/mL, respectively. After 48 h of incubation, the conditioned media (100 μL) were allowed to react with an equal volume of Griess reagent (Fluka, Steinheim, Germany) for 15 min at room temperature. The optical density was determined at 540 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA, USA). Nitric oxide production was evaluated via comparisons of the optical density with the standard curve obtained with sodium nitrite (Sigma, St. Louis, MO, USA).

Tolerance to artificial gastric juice and artificial bile acid

Tolerance to artificial gastric juice and artificial bile

acid was measured in accordance with the method developed by Kobayashi *et al*. (1974). The selected LAB strains were suspended in MRS broth containing 1%(w/v) pepsin (Sigma), adjusted to pH 2.5 with 0.1 M HCl, and incubated at 37°C for 2 h. Artificial bile acid tolerance was determined by cultivating cells treated with artificial gastric juice. The cells were incubated at 37°C for 24 h in artificial bile acid consisting of MRS broth containing 0.1%(w/v) oxgall (Difco Laboratories). The numbers of viable cells were determined by incubating aliquots for 24 h on MRS agar plates at 37°C.

Antimicrobial activities

The antimicrobial activities of selected LAB strains were assessed via a modified version of the deferred method against indicator organisms. Eight types of microorganisms (Avian pathogenic *E. coli* cell B/06/31, Avian pathogenic *E. coli* cell B/06/63, Avian pathogenic *E. coli* cell B/06/80, *E. coli* (-) control, *Staphylococcus aureus* SEA. CE. T-C, *Salmonella pullorum* ATCC 10398, *Salmonella gallinarum* ATCC 9184, and *Salmonella* Enteritidis ATCC 13076) were employed as the indicator strains. The pathogenic strains, isolated from chickens and hatcheries, were obtained from Dr. Hyung-Kwan Jang (Chonbuk National University, Korea). Overnight cultures in MRS broth were inoculated as 3 μL spots on MRS agar plates, and then incubated at 37°C for 24 h to allow for colony development. Five mL of soft TSA (0.75% agar), containing approximately 10^7 cells of indicator strains per overlay, were overlaid on MRS plates, and after 24 h of incubation at 37°C, an inhibition zone became clearly visible. The strength of the antimicrobial activities was expressed in terms of the diameter (mm) of the inhibition zone, and the results presented are the means of duplicate tests.

Antibiotic susceptibility

The antibiotics employed for the antibiotic susceptibility assay were nisin, streptomycin, neomycin, roxithromycin, chloramphenicol, gentamycin, rifampicin, erythromycin, ciprofloxacin, and ampicillin. Antibiotic susceptibility was determined via the paper disk method. Soft agar (0.75%, w/v), containing 10^7 cells of the selected LAB strains, was overlaid on agar plates. After solidification, sterile paper disks were aseptically laid onto the surface of the agar, and then antibiotic diluents were immediately applied to each disk. The agar plates with the antibiotic disks were then incubated at 37°C for 24 h. The inhibition zone was measured from the edge of the disk.

Results and Discussion

Screening of LAB from chicken feces

The use of LABs as probiotics in farm animals is increasing many researches including isolation, characterization and so on. The principal objective of this study was to screen and select LAB from chicken feces, feeds, and other sources, using acid tolerance characteristics. Forty six LAB isolates from chicken feces were screened for their acid tolerance (pH 3.5 for 2 h). Forty six colonies representing a variety of different colony morphologies were observed and randomly selected for further analysis. Selection was based on the collection of samples from different sites. The immunostimulatory effects of probiotics is an increasingly important characteristic reference. These isolates were then screened for immunostimulatory activity via nitric oxide production by *in vitro* culture experiments using RAW 264.7 murine macrophages. Among them, nine strains (FC113, FC222, FC322, FC421, FC422, FC511, FC621, FC721, and FC812) showed high NO production (Table 1). Strains FC812, FC222, and FC113 were shown to produce NO levels of 38.39±20.01, 35.06±27.73, and 33.88±15.99 μM, respectively, at concentrations of 10^8 CFU/mL. Thus, 9 LAB strains were selected for further analyses of probiotic characteristics.

Identification of LAB strains

Nine LAB strains were analyzed and tentatively identified via their physiological characteristics, which were determined using an API 50 CHL kit (data not shown). We tentatively identified strains FC113, FC222, FC421, FC422, FC511, FC621, FC721, and FC812 as *Lactobacillus salivarius*. Thus, these eight strains were tentatively named, respectively, *L. salivarius* FC113, FC222, FC421,

Table 1. NO productions of microbial strains isolated from chicken feces (μM)

Strain	Concentration of cells		
	10^6	10^7	10^8
FC113	0.35±1.56	12.51±0.90	33.88±15.99
FC222	3.10±1.48	14.86±2.93	35.06±27.73
FC322	1.53±1.66	13.10±3.34	23.49± 6.01
FC421	0.75±0.68	10.94±1.76	21.92± 6.48
FC422	4.27±2.38	15.45±1.36	16.24± 3.06
FC511	6.43±4.93	12.90±0.68	13.10± 3.24
FC621	8.39±4.79	21.14±7.02	23.29± 9.72
FC721	7.02±4.00	13.29±2.70	18.78± 3.24
FC812	6.63±0.90	20.16±4.90	38.39±20.01

Values are Mean±SE.

FC422, FC511, FC621, FC721, and FC812. FC322 could not be identified because no significant results were generated with an API 50 CHL kit.

Tolerance to artificial gastric juice and artificial bile acid

Probiotic bacteria must survive under gastric conditions in the stomach (low pH) in order to execute their various physiological functions. The viabilities of nine *Lactobacillus* strains were determined in artificial gastric juice (pH 2.5) for 2 h (Table 2). *L. salivarius* FC113, FC222, FC421, FC422, FC511, FC621, FC721, and FC812 evidenced high rates of survival (41-112%), whereas strain FC322 was the most sensitive under gastric conditions (1.4%). In particular, *L. salivarius* FC113 and FC522 evidenced survival rates of almost 100%. The results of similar studies showed that *L. acidophilus*, *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. bulgaricus*, *Lactococcus lactis*, and *Streptococcus thermophilus* are capable of growing well at low pH (Vinderola and Reinheimer, 2003; Xanthopoulos, 2000). *L. salivarius* was reported to survive for 30 min at pH 2 and for 6 h at pH 3 (Lim *et al.*, 2007), and to be a survival rate of approximately 50% after 2 h of incubation in artificial gastric juice (pH 3) (Park *et al.*, 1999).

Probiotics also need to be resistant to bile acids, as they pass through the duodenum in order to reach the small intestinal tract (Ha *et al.*, 2004; Mainville *et al.*, 2005). Table 2 shows the viability of nine *Lactobacillus* strains treated with artificial gastric juice in artificial bile acid for an incubation period of 24 h. *L. salivarius* FC222, FC511, and FC721 evidenced significantly higher survival rates (more than 70%) in artificial bile acid. However, *L. salivarius* FC113, FC621, and FC812, and strain FC322 were the least bile-resistant. The broad variation in bile sensitivity is consistent with the findings of many studies (Chateau *et al.*, 1994; Ibrahim and Bezkorovainy, 1993). Therefore, a consensus is emerging that broad variations exist in the susceptibility of probiotic bacteria to bile, and also that this property is specific as strain.

Antimicrobial activities against chicken-related pathogens

The modified deferred method was employed for the detection of the antimicrobial activities of the nine selected *Lactobacillus* strains against pathogenic microorganisms isolated from chickens and environmental specimens from hatcheries. All 9 strains of *Lactobacillus* evidenced antimicrobial activities, although the antimicrobial patterns they exhibited against the tested pathogenic microorganisms varied considerably (Table 3). In particular, *L. salivarius* FC422 and FC621 evidenced a broad range of antimicrobial activities against all tested pathogenic microorganisms. *L. salivarius* FC422 and FC621 produced a maximum zone of inhibition against *S.* Enteritidis 13676 and *S. gallinarum* 9184, respectively. *L. salivarius* FC113 and FC812 failed to evidence any detectable antimicrobial effects against APEC B/06/63 and *S. gallinarum* 9184, respectively. The growth of *S.* Enteritidis 13676 was inhibited effectively by all *Lactobacillus* strains. However, the least antimicrobial effects were noted against *S. aureus* SEA. CE. T-C. The antimicrobial activities of many LAB strains are principally attributable to lactic acid production, and the acid suppresses the contamination or growth of acid-sensitive intestinal pathogenic bacteria, including *Staphylococcus*, *Salmonella*, and coliform (Kim, 2005).

Antibiotic susceptibility

Probiotics must evidence some antibiotic tolerance, as many antimicrobial chemicals are employed as feed additives (Zhou *et al.*, 2005). LABs, in general, must be somewhat resistant to antibiotics, in order to survive in

Table 2. Viability of microbial strains isolated from chicken feces in artificial gastric juice for 2 h, and in artificial bile acid after artificial gastric juice treatment (for 2 h) for 24 h

Strain	Artificial gastric juice (CFU/mL)			Artificial bile acid (CFU/mL)		
	Control	1% Pepsin (pH 2.5)	Viability (%)	Control	0.1% Oxgall	Viability (%)
FC113	1.4×10^{8}	1.3×10^{8}	96.4	7.3×10^{9}	1.7×10^{7}	0.2
FC222	4.0×10^{8}	3.2×10^{8}	80.8	1.8×10^{10}	1.4×10^{10}	76.6
FC322	1.1×10^{8}	1.5×10^{6}	1.4	3.4×10^{7}	5.2×10^{4}	0.2
FC421	3.4×10^{8}	3.0×10^{8}	89.4	1.5×10^{10}	5.0×10^{9}	33.7
FC422	5.4×10^{8}	2.2×10^{8}	41.3	1.9×10^{10}	8.1×10^{8}	4.4
FC522	2.0×10^{8}	2.2×10^{8}	111.6	1.8×10^{10}	1.5×10^{10}	79.2
FC621	4.2×10^{8}	3.1×10^{8}	75.0	2.3×10^{10}	1.9×10^{8}	0.8
FC721	5.0×10^{8}	2.4×10^{8}	47.1	2.7×10^{10}	2.1×10^{10}	78.7
FC812	6.3×10^{8}	2.7×10^{8}	43.0	2.8×10^{10}	3.0×10^{8}	1.1

the intestine and allow for successful preventive antibiotic treatment. Antibiotics are important in the health care industry, in which they are used to fight bacterial infections. However, bacteria are capable of developing antibiotic resistance (Danielsen and Wind, 2003). All *Lactobacillus* strains were shown to be broadly resistant to streptomycin, and also resistant to nisin at concentrations below 200 μg/mL (Table 4). They also proved resistant to neomycin (except for strain FC322 and *L. salivarius* FC621), and gentamycin (except for *L. salivarius* FC222 and strain FC322). By way of contrast, they proved highly susceptible to rifampicin, chloramphenicol and ampicillin at 10

Table 3 Antimicrobial effect of microbial strains isolated from chicken feces against avian pathogens by deferred method

Strain	Diameter of inhibitory clear zone (mm)								
	FC113	FC222	FC322	FC421	FC422	FC511	FC621	FC721	FC812
APEC[1)] B/06/31	9	3	2	10	10	8	9	8	8
APEC B/06/63	0	3	3	10	10	14	10	8	11
APEC B/06/80	4	5	10	10	14	9	14	14	20
S. aureus SEA. CE. T-C	5	3	2	6	7	4	5	4	9
S. pullorum 10398-2	4	9	2	4	5	4	7	5	6
S. gallinarum 9184	10	9	3	8	8	8	50	12	0
S. Enteritidis 13676	10	30	6	10	70	18	20	14	16
E. coli (-) control	13	10	4	14	14	10	12	11	10

Avian pathogenic *E. coli* cell.

Table 4. Resistance of microbial strains isolated from chicken feces to various antibiotics

Antibiotics (μg/mL)		FC113	FC222	FC322	FC421	FC422	FC511	FC621	FC721	FC812
Nisin	10	+[1)]	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+
	200	+	-[2)]	+	-	+	+	-	-	+
Streptomycin	10	+	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+
Neomycin	10	+	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	+
	100	+	+	-	+	+	+	-	+	+
	200	+	+	-	+	+	+	-	+	+
Roxithromycin	10	+	+	+	+	+	+	+	+	+
	50	+	+	+	-	-	-	-	-	+
	100	-	+	+	-	-	-	-	-	+
	200	-	+	+	-	-	-	-	-	+
Chloramphenicol	10	-	+	+	+	+	+	+	+	+
	50	-	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-	-
Gentamycin	10	+	+	+	+	+	+	+	+	+
	50	+	+	-	+	+	+	+	+	+
	100	+	-	-	+	+	+	+	+	+
	200	+	-	-	+	+	+	+	+	+
Rifampicin	10	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-	-

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

Table 4. Conitnued

Antibiotics (μg/mL)		FC113	FC222	FC322	FC421	FC422	FC511	FC621	FC721	FC812
Erythromycin	10	+	+	+	+	+	-	+	+	+
	50	-	+	+	-	+	-	-	-	+
	100	-	+	-	-	+	-	-	-	+
	200	-	+	-	-	-	-	-	-	+
Ciprofloxacin	10	+	+	-	+	+	+	+	+	+
	50	+	+	-	+	+	+	+	+	+
	100	+	+	-	+	-	+	+	-	+
	200	-	+	-	+	-	+	+	-	+
Ampicillin	10	+	-	-	+	+	+	-	+	+
	50	-	-	-	-	-	+	-	-	-
	100	-	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-	-

μg/mL (*L. salivarius* FC511 at concentrations over 50 μg/mL). Roxithromycin inhibited *L. salivarius* FC113 at concentrations of over 50 μg/mL, and *L. salivarius* FC421, FC422, FC511, FC621, and FC721 at over 10 μg/mL concentration, whereas *L. salivarius* FC222, FC322, and FC812 did exhibit resistance. *L. salivarius* FC222 and FC812 proved resistant to erythromycin; moreover, *L. salivarius* FC422 was also resistant to erythromycin at concentrations below 200 μg/mL. However, erythromycin inhibited *L. salivarius* FC511 and *L. salivarius* FC113, FC421, FC621, and FC721 at concentrations of over 10 μg/mL, as well as *L. salivarius* FC322 at concentrations above 50 μg/mL. All *Lactobacillus* strains, with the exception of strain FC322, evidenced ciprofloxacin resistance (*L. salivarius* FC113 at concentrations below 200 μg/mL, and *L. salivarius* FC422 and FC721 at concentrations below 100 μg/mL).

All in all, our results led us to the conclusion that *L. salivarius* strains have probiotic properties, including NO production, marked resistance to artificial gastric juice and bile acid, antimicrobial activities, and antibiotic tolerance. These findings demonstrate that these probiotic organisms evidence potential probiotic properties as an alternative to antibiotic use in animals. However, strain FC322 evidences poor probiotic characteristics, particularly low survival rates under gastric conditions.

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