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Intestinal Colonization Characteristics of *Lactobacillus* spp. Isolated from Chicken Cecum and Competitive Inhibition Against *Salmonella typhimurium*

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Abstract Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microflora. To select the best *Lactobacillus* spp. as a chicken probiotic, probiotic characteristics of 10 selected *Lactobacillus* strains isolated from chicken cecum or obtained from KCTC were investigated. The strains were examined for resistance to pH 2.0 and 0.3% oxgall, and adhesion to cecal mucus and cecal epithelial cells. All strains grew in MRS containing 0.3% oxgall. However, *Lb. plantarum* AYM-10, *Lb. fermentum* YL-3, AYM-3, and *Lb. paracasei* YL-6 showed relatively high resistance to 0.3% oxgall. *Lb. fermentum* YL-3, YM-5, AYM-3, and *Lb. paracasei* YL-6 survived 4 hours of incubation at pH 2.0. *Lb. fermentum* YL-3, KCTC 3112, and *Lb. plantarum* AYL-5 were strongly adhesive to cecal mucus, while the rest showed moderate or low adhesion. *Lb. plantarum* AYM-10, AYL-1, and AYL-5 had good adhering properties to cecal epithelial cells (30.7±10.82, 40.2±20.90, and 14.5±4.22, respectively). *Lb. fermentum* YL-3, AYM-3, and KCTC 3547 showed intermediate adhesion ability, and *Lb. plantarum* showed better adhesion ability to cecal epithelial cells than *Lb. fermentum*. Attached *Lb. fermentum* YL-3 to cecum after 60 min incubation was confirmed using CLSM. *Lb. fermentum* YL-3 attached to a matrix which was composed of a mucus layer adjacent to intracrypts and pericryptal region. Some *Lb. fermentum* YL-3 bound to mucosal epithelial cells. From these results, *Lb. fermentum* YL-3 was selected as a chicken probiotic. *In vivo* trials of chicks inoculated with *Lb. fermentum* YL-3 had decreased *Salmonella* population in cecal contents and livers ($p<0.5$).

Key words: Probiotics, *Lactobacillus*, CLSM, adhesion, mucus, acid tolerance, bile tolerance

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The beneficial effects of probiotics are mainly attributed to direct antagonistic effect against specific groups of microoganisms (Enteropathogens), by either an effect on the metabolism in the gut or by a stimulation of systemic or mucosal immunity.

The most commonly used organisms in probiotic preparations are the lactic acid bacteria (lactobacilli, streptococci, and bifidobacteria). They are found in large numbers in the gut of healthy animals and do not appear to affect them adversely. Members of the genus *Lactobacillus* are most commonly given safe or generally recognised as safe (GRAS) status. However, members of the genera *Streptococcus* and *Enterococcus* contain many opportunistic pathogens [14].

In chickens, *Salmonella* are the main pathogenic microorganisms that colonize the intestinal tract. The infection does not cause serious illness in birds and production losses are not normally very important, however, *Salmonella* in chicken is the main source of pathogens for humans. The extensive use of antibiotics in animal farms with the purpose of promoting growth rate and increasing feed conversion efficiency, and for the prevention of intestinal infections, has led to an imbalance of the beneficial intestinal flora and the appearance of resistant bacteria. There has been a continuing dialogue on the impact of antibiotic use in animals on resistance emergence in zoonotic pathogens, such as *Salmonella*, *Campylobacter*, and *E. coli*, found in the intestinal tracts of animals [15, 35, 36] and its implication for the effectiveness of antibiotic therapy in human infections [4, 13, 20, 21, 29, 32].

The use of probiotics in order to competitively exclude the colonization of intestinal pathogens has been proposed for poultry. A great number of studies have demonstrated that providing newly hatched chicks with intestinal microflora from adult chickens decreases the incidence of salmonellae cecal colonization [1, 2, 9, 10, 26, 27, 30, 33, 34].

The purposes of this study were to select the best probiotic strain and to evaluate the protective effects of the selected strain on *Salmonella* colonization in broiler chicks.

MATERIALS AND METHODS

Bacterial Strains

This study comprised 10 strains of *Lactobacillus* spp. (Table 1), which were isolated from healthy chicken cecum in a laboratory [6] and obtained from the Korean Collection for Type Cultures (KCTC). *Salmonella typhimurium* KCTC 2515 was obtained from the KCTC.

Acid Tolerance and Bile Tolerance

Lactobacillus spp. were cultured in MRS broth at 37°C for 24 h. Bacterial cultures were centrifuged at 1,000 ×g for 10 min at 4°C. The bacteria were washed once with phosphate buffered saline (PBS) and resuspended in PBS. Bacteria ($\times 10^9$ CFU/ml) were inoculated in 9 ml of MRS broth (pH 2.0) and incubated at 41°C, and surviving cells were counted on MRS agar following 0, 1, 2, 4, and 6 h.

The bile tolerance test was performed in flat bottom 96-well microplates (Nunc,Naperville, U.S.A.). Three hundred μl volume of MRS (BBL) containing 0.3% oxgall or normal MRS were inoculated with the bacteria at a level of 10^6 CFU/ml, and broth without inoculation was used as a control. Changes in optical density at 600 nm were measured (Microplate Reader, Benchmark, Bio-Rad, Hercules, U.S.A.) following 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 12, 18, and 24 h of incubation at 41°C.

Adhesion Assay

Radioactive Labeling of *Lactobacillus* spp. Cultures were grown for 24 h at 37°C on MRS broth containing 0.01 mCi of [2, 8-^{3}H] adenine (Sigma, St. Louis, U.S.A.) per ml. Cells from labeled cultures were washed twice with HEPES-Hank's buffer (pH 7.4) by centrifuging at 1,000 ×g for 15 min at 4°C, and resuspended in buffer.

Table 1. *Lactobacillus* spp. used in this study.

Strains	Source
Lactobacillus fermentum YL-3	Chicken cecum
Lactobacillus fermentum YL-10	Chicken cecum
Lactobacillus fermentum YM-5	Chicken cecum
Lactobacillus fermentum AYM-3	Chicken cecum
Lactobacillus paracasei YL-6	Chicken cecum
Lactobacillus plantarum AYM-10	Chicken cecum
Lactobacillus plantarum AYL-1	Chicken cecum
Lactobacillus plantarum AYL-5	Chicken cecum
Lactobacillus fermentum KCTC 3547	Saliva
Lactobacillus fermentum KCTC 3112	Fermented beets

Cecal Mucus and Epithelial Cell Preparation. Cecal mucus and epithelial cells were prepared from chick cecum according to the method of Craven *et al.* [11]. Briefly, the cecum were removed from 2-6-week-old chicks. Contents were removed by gentle pressure and the mucosal surfaces were rinsed with 0.01 M PBS (pH 7.4). The mucus was removed from the walls of excised cecum by gently scraping it into HEPES-Hank's buffer. Cecal epithelial cells and membranes were separated from mucus by centrifuging twice at 12,000 ×g for 15 min at 4°C, and once at 27,000 ×g for 15 min at 4°C. After the mucus was removed, the excised cecum were incubated for 30 min at 37°C in 20 ml of a solution containing 1 mg of hyaluronidase (Sigma) per ml of HEPES-Hank's buffer, and the cecal epithelial cells were removed by gentle scraping. After removal, it was filtered through nylon stocking to remove clumps and debris. The cecal epithelial cell suspension was centrifuged at 100 ×g for 1 min and the pellet was washed by centrifuging (at 120 ×g, for 10 min) twice in 5 ml and once in 15 ml of HEPES-Hank's buffer (pH 7.4).

Cecal Mucus Immobilization and Adhesion Assay. Cecal mucus was adjusted to a protein concentration of 500 μg per ml of HEPES-Hank's buffer using the Bio-Rad Protein Assay and bovine serum albumin (BSA) as a protein standard, and added (0.2 ml per well) to 24-well tissue culture plates. The plates were incubated overnight at 4°C to allow the cecal mucus to bind to the plates. The plates were incubated for an additional 1 h at 37°C and washed twice with 0.5 ml of HEPES-Hank's buffer. Unbound sites were blocked by adding 0.2 ml of 1% bovine serum albumin in HEPES-Hank's buffer per well and by incubating for 1 h at 37°C, and then rinsed twice with buffer. Tritium-labeled *Lactobacillus* spp., washed and suspended in HEPES-Hank's buffer, was added to each well. The plates were incubated for 1 h at 37°C, and were then washed twice with 0.5 ml buffer to remove nonadhering bacteria. To recover attached bacteria, 500 μl of 5% sodium dodecyl sulfate (SDS) solution was added to each well and incubated at 37°C for 3 h. The samples were collected and mixed thoroughly with 4.5 ml aquasol (Packard, Salt Lake, U.S.A.). The radioactivity of the SDS-extracted sample was enumerated using a Packard 1500 CA Tri-carb liquid scintillation analyzer.

Cecal Epithelial Cell Adhesion Assay. Cecal epithelial cell and bacteria (*Lb.* spp) were mixed in a final volume of 1 ml and incubated for 40 min at 4°C. Suspensions were then centrifuged (200 ×g for 15 min) and washed three times with HEPES-Hank's buffer. The pellet was resuspended to half of the initial volume and smeared on the slide glasses [37]. The cells and bacteria were then fixed with methyl alcohol and stained with crystal violet (30 sec) and iodine (60 sec), destained with 95% ethanol, and stained with safranin (30 sec). Attached bacteria were examined by light microscopy, and an adhesion index was calculated as

a mean value of the number of bacteria attached to approximately 20 cells.

Confocal Laser Scanning Microscopy

Lb. fermentum YL-3 cultured overnight were centrifuged (1,000 ×g for 10 min) and washed. Chick ceca fixed in 10% neutral formalin were washed 60 min before use. Ceca were incubated with bacteria for 60 min at 37°C. Ceca and bacteria were stained with cyto 9 and propidium iodine. Stained ceca and bacteria were immediately examined by confocal laser scanning microscopy (Bio-Rad MRC-1024, Hercules, U.S.A.).

***In Vivo* Experiment**

Chicks and Feeds. One-day-old broiler chicks were purchased from a commercial hatchery, randomly divided into three treatment groups (*Lactobacillus*, *Lactobacillus*+ *Salmonella*, *Salmonella*) and a control group of 20 chicks. They were housed in electrically heated chick isolators (Three Shine, Seoul, Korea) controlled at 35±2°C, with 60±10% relative humidity and righted throughout the experimental period. Chicks were fed water and modified feed *ad libitum*. The feed (Table 2) was a balanced unmedicated corn-soybean meal-based ration that met or exceeded requirements recommended by National Research Council in the U.S.A. [25]. Ingredients for the feed were provided by Samyang feed company.

Preparation of Bacteria. *Lb. fermentum* YL-3 incubated for 24 h at 37°C were centrifuged at 1,000 ×g (15 min, 4°C) and the bacteria resuspended and adjusted to 5×10^8 CFU/ml. *Salmonella typhimurium* was selected for testing resistance to novobiocin (NO) and nalidixic acid (NA) in our laboratory and maintained in media containing 25 μg of NO and 20 μg of NA per ml. Challenge inoculation was prepared from an overnight culture which had previously been transferred three times in trypticase soy broth (Difco, Maryland, U.S.A.) containing NO and NA, and then serially diluted in sterile phosphate buffered saline to a concentration of 4×10^4 CFU/ml.

Table 2. Basal diet composition.

Ingredients (%)	Percentage
Corn yellow	59.68
Soy bean meal	28.96
Fish meat	5.00
Animal fat	3.81
Calcium phosphate	1.70
Methionine	0.24
Limestone	0.25
Vitamin premix	0.20
Salt	0.16
Total	100.00

Vitamin premix ingredients per kg

Vit. A-10,000,000 IU, Vit. D3-1,800,000, Vit. E-10,000, Vit. K3-2,000 mg, Vit. B2-4,400 mg, Pantothen-9,000 mg, Niacin- 25,000 mg, Biotin-30 mg, Folic acid-200 mg, Vit. B12-18 mg, Ethoxyquin-50,000 mg.

Experimental Design. On day 1, the chicks were inoculated with 0.5 ml of the suspension of *Lb. fermentum* YL-3 (5×10^8 CFU/ml) using a rubber zonde needle. On day 3, ten chicks from each group were euthanatized by cervical dislocation and cecal contents from each chick were collected aseptically and evaluated for *Lactobacillus* spp. colonization and pH. The chicks were challenged with 0.5 ml of the suspension of NO-NA-resistant *Sal. typhimurium* (4×10^4 CFU/ml). After seven days, ten chicks from each groups were euthanatized by cervical dislocation and cecal contents from each chick were collected aseptically and evaluated for *Lactobacillus* spp. and *Salmonella typhimurium* colonization and pH.

Bacteriological Analysis. One cecum from each chick was removed aseptically, minced with scissors, incubated in 5 ml of selenite-cystine broth for 24 h at 37°C, and examined for typical *Salmonella* colonies. Typical *Salmonella* colonies were confirmed by biochemical tests on triple sugar iron agar. A portion of the contents of the remaining cecum (0.2 g) was serially diluted in PBS to 1:100, 1:1,000, and 1:10,000. Diluents were spread-plated on NO and NA brilliant green agar (BGA) plates to determine *Salmonella* colonization. The plates were incubated at 37°C for 24 h, and the number of NO and NA-resistant *Sal. typhimurium* colonies per g of cecal content were determined on a colony counter. *Salmonella* CFU were expressed as log10 of *Salmonella*. Culture-negative at the 1:100 dilution but positive after culture in selenite-cystine broth were arbitrarily assigned a value of 1.5 $\log_{10}$ *Salmonella* per g of cecal contents. Selenite-cystine cultures that were negative on BGA plates were assigned a log10 *Salmonella* value of 0. For determining *Lactobacillus* spp. colonization, diluents (1:10,000, 1:100,000, and 1:1,000,000) were spread-plated on LBS agar (BBL, Cockeysville, U.S.A.) plates, the plates were incubated at 37°C for 24 h, and the number of colonies per g of cecal content were determined on a colony counter.

Measurement of Cecal pH. Cecal contents (0.2 g) were collected from each chick, suspended in 0.8 ml of sterile distilled water, and pH was immediately measured with an IQ2000 pH meter (IQ Scientific Instruments, Sandiego, U.S.A.).

Statistical Analysis. Differences among treatments were examined for level of significance by the General Linear Models Procedure and Duncan's Multiple Range Test using PCSAS release 6.02 (SAS Institute, Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Numerous studies suggest desirable effects of probiotic lactobacilli on the health and performance of poultry.

Table 3. Adhesion properties to epithelial cells, and pH and bile tolerances.

Strains	Adhesion index[a]	Growth/survival[b]	
		pH 2.0	0.3% oxgall
Lb. fermentum YL-3	++	-/+	***/+
Lb. fermentum YL-10	+	-/-	**/+
Lb. fermentum YM-5	+	-/+	**/+
Lb. fermentum AYM-3	++	-/+	***/+
Lb. paracasei YL-6	++	-/+	**/+
Lb. plantarum AYM-10	++++	-/-	***/+
Lb. plantarum AYL-1	++++	-/-	**/+
Lb. plantarum AYL-5	+++	-/-	*/+
Lb. fermentum KCTC 3547	+	-/+	**/+
Lb. fermentum KCTC 3112	++	-/-	**/+

[a]Adhesion index was expressed as the number of attached bacteria per cell. (+ : <5, ++ : 5-10, +++ :11-15, ++++ : >16).
[b]***growth delay of <2 h; **growth delay of < 4 h; *, growth delay of >4 h; -, no growth or survival.

The present study was carried out to select the best strain as a chicken probiotic and assess the competitiveness of a selected probiotic strain, *Lb. fermentum* YL-3, for rearing of chicks with the ability to minimize *Salmonella* colonization.

Acid and Bile Tolerance

Lb. fermentum YL-3, YM-5, AYM-3, and *Lb. paracasei* YL-6 have higher viability in MRS broth (pH 2.0) than the other strains obtained from the KCTC. The results in Table 3 showed that *Lb. fermentum* YL-3 survived 6 h of incubation at pH 2.0. All the strains have bile tolerance.

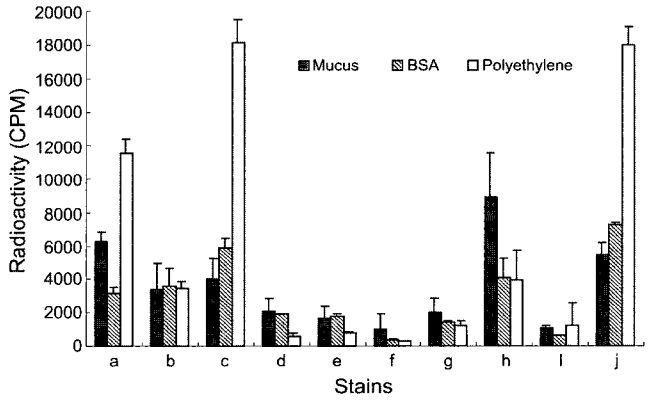

Fig. 1. Adhesion of *Lactobacillus* spp. to cecal mucus, BSA, and polystyrene.
Results are expressed as the mean of radioactivity (CPM). Bar markers=S.D. a, *Lactobacillus fermentum* YL-3; b, *Lactobacillus fermentum* YL-10; c, *Lactobacillus fermentum* YM-5; d, *Lactobacillus fermentum* AYM-3; e, *Lactobacillus paracasei* YL-6; f, *Lb. plantarum* AYM-10; g, *Lactobacillus plantarum* AYL-1; h, *Lactobacillus plantarum* AYL-5; I, *Lactobacillus fermentum* KCTC 3547; j, *Lactobacillus fermentum* KCTC 3112.

Lb. plantarum AYM-10, *Lb. fermentum* YL-3, AYM-3, and *Lb. paracasei* YL-6 showed relatively high resistance to 0.3% oxgall. Tolerance to the low pH of the stomach and bile of upper parts of the intestines, and the ability to colonize intestinal tract seem to be very important. The primary mechanism for maintaining pH by microoganisms is dependent upon the expulsion of protons from cytoplasm by H^+-ATPase at the expense of ATP [19, 24, 28].

Adhesion Ability

One factor that has been generally used as a selection criterion for probiotic formulation is the ability of strains to adhere to the hosts' gut epithelia. There exists a good correlation between *in vitro* and *in vivo* results for bifidobacterial strains adhesion [12]. In this study, *Lb. fermentum* YL-3, KCTC 3112, and *Lb. plantarum* AYL-5 were strongly adhesive to cecal mucus, while the rest showed moderate-to-low adhesion (Fig. 1). Various cell wall components of *Lactobacillus* have been proposed to have affinity for squamous nonsecreting epithelial cells, e.g. acidic mucopolysaccharide [5], proteins [8], and lipoteichoic acid [31].

Lb. plantarum AYM-10, AYL-1, and AYL-5 have good adhering properties to epithelial cells (Table 3). *Lb. fermentum* YL-3, AYM-3, and KCTC 3112 showed intermediate adhesion ability, and *Lb. plantarum* showed better adhesion ability to cecal epithelial cells than *Lb. fermentum*. Adhesion of lactobacilli to intestinal epithelial cells, a model for the adhesion to piglet ileal epithelial cells, has been less well studied. As an adhesion model of *Lb. acidophilus* to human intestinal cells, involvement of a proteinaceous component has also been proposed by Coconnier *et al.* [7]. The ability to adhere may be an important direct or indirect factor in the ability of lactobacilli to reduce intestinal colonization by other bacteria.

In order to influence the immune system, a probiotic microoganism must activate the lymphoid cells of the gut-associated lymphoid tissue (GALT), which are diffusely distributed among epithelial cells and populate the lamina propria and sub-mucosa [23]. *Lb. fermentum* YL-3 attached to cecal epithelial cells adjacent to intracrypts and pericryptal region were confirmed using confocal laser scanning microscopy (CLSM) in this study. This may cause a transient translocation of small numbers of bacteria via M cells of the Peyer's patches and other GALT surfaces. Therefore, at least the immune responses generated in response to metabolites produced probiotics *in situ*, and the indirect mechanisms might be related to their additional pathways.

In Vivo Competitive Inhibition Against *Sal. typhimurium*

The newly hatched chicks inoculated with *Lb. fermentum* YL-3 had decreased cecal colonization of *Sal. typhimurium*

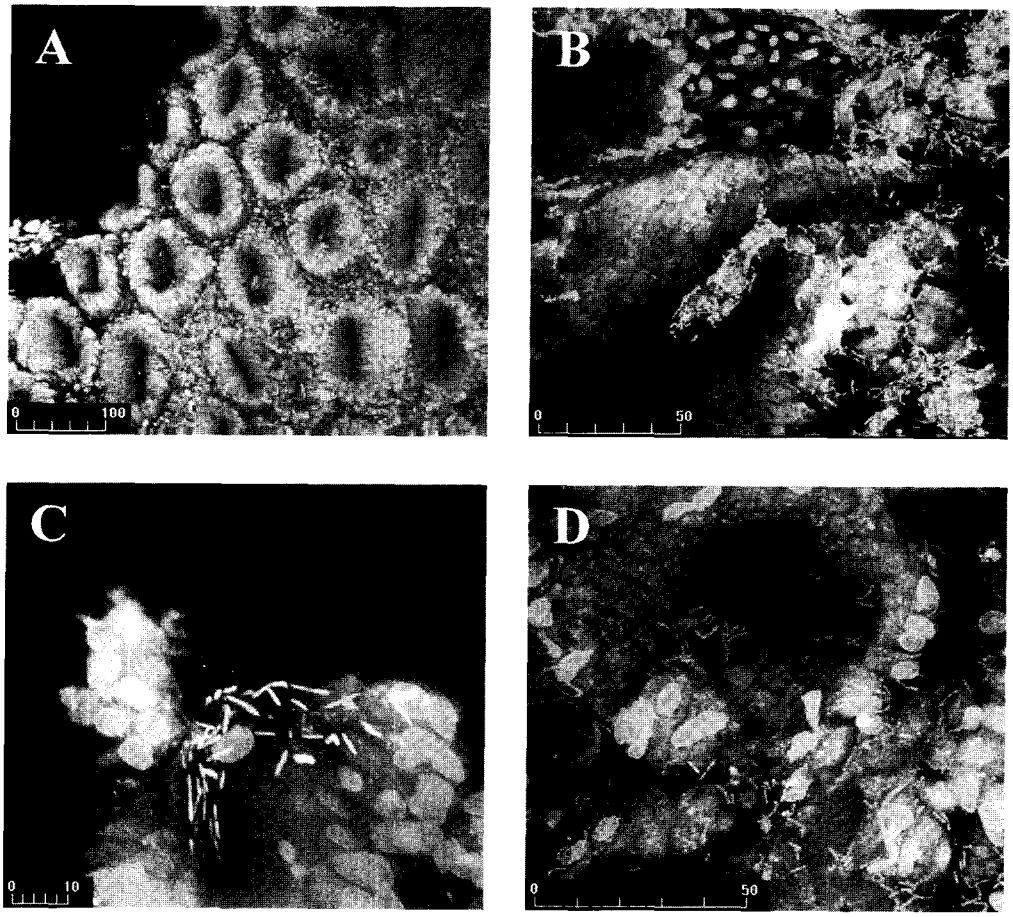

Fig. 2. Confocal laser scanning micrographs of cecum after 60 min incubation with *Lb. fermentum* YL-3.
A number of crypts were observed in chick cecum (A). *Lb. fermentum* YL-3 attached to mucus layer adjacent to intracrypts and pericryptal region. Also attached to the region of inner crypt (B, C, D). Bar scale: μm.

by 1.02 or 0.15 $\log_{10}$ units, respectively, and transmission of *Sal. typhimurium* into liver decreased significantly ($p<0.05$) by 0.87 $\log_{10}$ units (Tables 4, 5). *Lactobacillus* spp. were decreased significantly ($p<0.05$) in the group inoculated only with *Sal. typhimurium*. Various theories have been proposed as to the mechanisms by which normal intestinal flora protect their host against invading enteropathogens. They include competition for limiting nutrients [16, 17, 18], competition for attachment sites on the intestinal mucosa [22, 27, 33], and production of short-chain bacteriostatic volatile fatty acids, particularly acetic, propionic, and butyric acids [22, 1, 30].

Table 4. *In vivo* trial I : Competitive inhibition of *Lb. fermentum* YL-3 against colonization of *Sal. typhimurium*.

	3 day		10 day		
	Cecum		Cecum		
	pH[a]	*Lb.* spp[b]	pH[a]	*Lb.* spp[b]	*Sal.*[b]
Group 1 (*Lb.*)	5.59±0.342[a]	6.22±1.58[a]	6.13±0.392[a]	8.09±0.44[a]	ND[c]
Group 2 (*Lb.*+*Sal.*)	5.34±0.340[a]	6.89±0.73[a]	6.02±0.450[a]	7.45±1.84[a]	4.82±1.91[a]
Group 3 (*Sal.*)	5.11±0.199[a]	>5	5.39±0.250[b]	4.68±1.91[b]	5.81±1.27[a]
Group 4 (con.)	5.19±0.348[a]	>5	5.57±0.264[b]	7.51±1.13[a]	ND

[a]Values are means±standard deviations. Means within the same column with different superscripts differ significantly ($p<0.05$).
[b]Values represent the mean $\log_{10}$ CFU (±standard deviation) per gram of cecal content. Means within the same column with different superscripts differ significantly ($p<0.05$).
[c]ND, not determined

Table 5. *In vivo* trial II : Competitive inhibition of *Lb. fermentum* YL-3 against colonization of *Sal. typhimurium*.

	3 day		10 day			
	Cecum		Cecum			Liver
	pH[a]	*Lb.* spp[b]	pH[a]	*Lb.* spp[b]	*Sal.*[b]	*Sal.*[b]
Group 1 (*Lb.*)	5.26±0.224[a]	7.23±0.66[a]	5.49±0.291[a]	6.33±0.62[a]	ND[c]	ND
Group 2 (*Lb.*+*Sal.*)	5.12±0.196[a]	7.43±1.50[a]	5.62±0.227[a]	5.62±1.80[a]	2.59±2.20[a]	1.59±0.99[a]
Group 3 (*Sal.*)	5.30±0.509[a]	7.06±1.25[a]	5.68±0.237[a]	3.81±1.75[b]	2.74±2.09[a]	2.59±0.86[b]
Group 4 (con.)	5.16±0.336[a]	7.02±0.90[a]	5.50±0.176[a]	6.01±2.00[a]	ND	ND

[a]Values are means±standard deviations.

[b]Values represent the mean $\log_{10}$ CFU (±standard deviation) per gram of cecal content. Means within the same column with different superscripts differ significantly ($p<0.05$).

[c]ND, not determined.

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