British Poultry Science Volume 53, Number 2 (April 2012), pp. 183–189

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In vitro screening of lactobacilli isolated from chicken excreta to control *Salmonella* Enteritidis and Typhimurium

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Abstract 1. The aim of this work was to select lactic acid bacteria (LAB) strains from chicks and hens of egg-laying strains for potential use to control *Salmonellae*.
2. Nineteen LAB strains obtained from culture collections, and 24 strains isolated from excreta of laying hens and chicks, were evaluated for inhibitory capacities against two *Salmonella* serotypes using a ''Spot-the-lawn'' technique and other *in vitro* properties that could be predictive of antimicrobial activity.
3. The size of the inhibition zone differed slightly between *Salmonella* serotypes, however, the mean size of the *Salmonella* inhibition zone differed greatly among the LAB strains. *Lactobacillus salivarius, L. plantarum, L. rhamnosus* and *L. reuteri* exhibited powerful inhibitory effects to each *Salmonella* strain.
4. The result of the acid tolerance test showed that all *L. salivarius, L. kitasatonis* strains and each of *L. ingluviei* cannot survive in a low pH environment. In the bile acid tolerance assay, growth was inhibited in all strains, except *L. kitasatonis* HE4, and a large inhibition was observed in most of the *L. salivarius* and *L. crispatus* strains.
5. The results demonstrate that some LAB of poultry origin were able to inhibit the growth of *Salmonella* and survive simulated passage through the gastrointestinal tract. The selected LAB could act in the lower gastrointestinal tract to prevent salmonellosis in poultry.

INTRODUCTION

During the past two decades, the incidence of food poisoning caused by *Salmonella* infections in humans has increased in Japan (Ministry of Health, Labour and Welfare, 2009) and contaminated eggs have been thought to be an important source of infection. The use of *Lactobacilli* has been suggested as an effective strategy to reduce *Salmonella* infection in laying hens (Gusils *et al.*, 1999; Van Coillie *et al.*, 2007). *Lactobacilli* have been shown to inhibit the growth of various bacterial pathogens by the production of organic acids and specific inhibitory proteins called bacteriocins (Jin *et al.*, 1996) that inhibit adherence of *Salmonella* to the epithelium by competitive adhesion, and by stimulating the host immune function.

Control of *Salmonellae* with caecal microorganisms has been largely achieved by the use of competitive exclusion (CE), a concept originally described by Nurmi and Rantala (1973). At present, most commercially available CE products have been prepared from mixed bacterial cultures originating from the caecal contents of adult chickens. However, undefined CE products involve the risk of transferring opportunistic pathogens from the poultry microbiota. To prevent this risk, the use of a defined microorganism product, containing a well-defined strain that inhibits the growth and invasion of pathogens, like *Salmonella*, is a better policy.

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Accepted for publication 18th October 2011.

ISSN 0007–1668(print)/ISSN 1466–1799 (online)/12/0020183–7
http://dx.doi.org/10.1080/00071668.2012.678814

The objectives of the current studies were to test a number of *Lactobacilli*, which were purchased from a microorganism bank or isolated from the excreta of chicks and laying hens, for their inhibitory capacities against two *Salmonella* serotypes.

MATERIALS AND METHODS

Bacterial strains

This study comprised 43 strains of LAB (Table 1), of which 19 were purchased from the Japan Collections of Microorganisms (JCM), American Type Culture Collection (ATCC), and LQ80 (*L. plantarum*) provided by Dr Yimin Cai (National Institute of Livestock and Grassland Science, Japan). Another 24 strains were isolated from the excreta of laying hens and chicks. White Leghorn laying hens (100 weeks old), and crossbred White Leghorn males and Rhode Island Red female chicks (two weeks old) were housed in battery cages. Each bird had free access to water and a commercial diet. Cecum and non-cecum contents were collected immediately after excretion and dissolved in sterilized PBS solution. The solution was diluted in PBS and plated on to deMan Rogosa Sharpe (MRS) agar and incubated for 48 or 72 h at 37°C. The colonies were further purified on MRS agar, and stored at −80°C in MRS broth containing 10% glycerol.

Strain identification

The isolated LAB strains were identified with the 16S rRNA gene sequence. Total DNAs were extracted from the strains using the protocol described by Johnson (1991). Amplification of the 16S rRNA gene was generated by PCR using a pair of 27 forward and 1525 reverse universal primers (Lane, 1991). PCR was conducted in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using Takara ExTaq HS (Takara Bio Inc., Ohtsu, Japan) in accordance with the manufacturer's instructions. PCR was performed as follows; initial denaturation at 95°C for 10 min, 30 cycles at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 60 sec, with the final elongation at 72°C for 7 min. The PCR products were purified with an ExoSAP-IT kit (GE Healthcare Bio-Science Corp., NJ, USA) and used as templates for the sequencing reaction with BigDye ver. 3·1 (Applied Biosystems, CA, USA). The same primer pair was used for the sequencing reaction. Partial sequences of the 16S rRNA gene were read on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The sequences obtained from each strain were assembled with Geneious Pro ver. 5·0·2 (Biomatters Ltd., Auckland, New Zealand). A sequence similarity search against database entries was done using on-line BLAST (Altschul *et al.*, 1997). The length of high-quality sequences produced was usually about 1100 bp. Nucleotide sequences from the strains were deposited in the GenBank under the accession numbers AB596980-AB597003.

Inhibition assay 1 (spot-the-lawn technique)

The inhibitory effect of the LAB strains against each of two *Salmonella enterica* strains (*S. Enteritidis*: NIAH 12175 and *S. Typhimurium*: NIAH 12186) was assessed through the "spot-the-lawn technique" (Oyarzabal and Conner, 1995). LAB strains were grown at 37°C for 48 h in MRS broth. Subsequently, 10 µl of the liquid culture was dispensed on the top of a 6 mm diameter paper disk (Nihon Becton, Dickinson and Company Inc., Tokyo, Japan). For the negative control, 10 µl of MRS broth was dispersed. After air drying, the paper disks were placed on an MRS agar plate, and incubated at 37°C for 24 h. Subsequently, 9 ml of trypticase soy soft agar (0·6%) was inoculated with 0·5 ml of *Salmonella* culture broth (incubated 24 h in trypticase soy broth) and poured over the plates. The plates were subsequently incubated at 37°C for 24 h. The radius of the clear zone around the paper disks was determined, and taken as a measure of *Salmonella* inhibition.

Inhibition assay 2 (detection of antimicrobial activity)

The supernatant of each LAB culture was incubated with *Salmonella* for 4 h, and growth of *Salmonella* was assessed with a turbidimeter (Miniphoto 518R, Taitec Co., Ltd., Koshigaya, Japan) at a wavelength 660 nm. LAB strains to be tested were cultured overnight in the MRS broth at 37°C. Cultures were centrifuged at 10,000 g for 30 min at 4°C and supernatant fluid was collected and filtered (Advantec DISMIC-25CS, 0·45 µm pore size) to remove any remaining bacteria. Filtered supernatant were divided into two, one was unadjusted and the other was adjusted to pH 6·25 with NaOH. Cells of *S.* Enteritidis and Typhimurium from an overnight culture in trypticase soy broth were pelleted at 3,700 g for 20 min at 4°C and supernatant was discarded. Bacteria were washed twice with sterilized PBS solution and resuspended in trypticase soy broth to a density of 1×10^9 colony forming units (CFUs)/ml. The assay was performed by incubating 1 ml of this suspension with 1 ml of LAB supernatant (unadjusted or adjusted to pH 6·25 with NaOH) at 37°C for 4 h. Control experiments were performed by incubating the same amount of *Salmonella* broth with MRS broth medium

Table 1. *Inhibitory capacities of LAB strains against* Salmonella *spp*

Strain	Growth inhibition (mm)		Relative turbidity, %[1]			
			Intact		Adjusted to pH 6·25	
	SE[2]	ST[3]	SE[2]	ST[3]	SE[2]	ST[3]
Lactobacillus delbrueckii JCM 1012	3·0 ± 0·9[4]	3·9 ± 1·5	74·8 ± 2·5	83·1 ± 1·8	142·8 ± 1·2	122·0 ± 2·5
Lactobacillus delbrueckii JCM 1248	1·3 ± 0·3	3·3 ± 3·6	106·2 ± 0·4	101·7 ± 1·3	230·3 ± 2·1	157·1 ± 1·3
Lactobacillus intestinalis JCM 7548	1·3 ± 0·4	2·3 ± 1·1	−24·2 ± 7·6	−28·8 ± 3·6	179·8 ± 15·2	173·0 ± 3·6
Lactobacillus amylovorus JCM 1126	9·0 ± 1·7	9·2 ± 0·8	69·8 ± 0·3	79·6 ± 5·6	136·6 ± 1·4	124·0 ± 1·9
Lactobacillus paracasei JCM 1181	9·4 ± 1·3	9·7 ± 1·5	49·3 ± 4·2	62·9 ± 3·6	174·6 ± 22·5	172·7 ± 4·1
Lactobacillus manihotivorans JCM 12514	1·7 ± 0·5	2·3 ± 0·8	76·1 ± 2·1	79·1 ± 1·8	126·8 ± 1·4	111·3 ± 3·4
Lactobacillus brevis JCM 1059	6·9 ± 0·9	8·2 ± 1·6	72·1 ± 0·9	75·6 ± 1·5	132·3 ± 2·3	119·2 ± 4·1
Lactobacillus johnsonii JCM 2012	3·8 ± 1·8	4·6 ± 2·0	−23·7 ± 5·2	−31·5 ± 2·1	172·9 ± 21·7	171·5 ± 3·6
Lactobacillus fermentum JCM 1173	8·8 ± 1·8	9·4 ± 0·5	52·2 ± 2·8	66·3 ± 2·3	130·7 ± 1·6	115·6 ± 2·9
Lactobacillus reuteri JCM 1112	5·8 ± 1·1	8·4 ± 1·5	5·5 ± 0·6	6·2 ± 1·4	117·5 ± 1·2	113·2 ± 1·3
Lactobacillus plantarum JCM 1149	11·2 ± 0·7	10·8 ± 0·8	−5·4 ± 4·5	13·1 ± 4·6	190·0 ± 8·1	176·0 ± 5·4
Lactobacillus animalis JCM 5670	5·3 ± 0·6	5·9 ± 0·7	8·7 ± 0·5	10·7 ± 0·2	98·4 ± 2·2	97·8 ± 1·6
Lactobacillus gasseri JCM 1131	9·3 ± 0·6	9·0 ± 1·2	1·6 ± 0·7	1·6 ± 0·7	132·9 ± 2·0	114·3 ± 3·0
Lactobacillus paracasei JCM 8130	7·8 ± 1·1	8·4 ± 1·3	64·1 ± 1·2	69·7 ± 2·9	130·8 ± 4·5	113·0 ± 0·7
Lactobacillus casei JCM 1134	8·6 ± 0·5	9·2 ± 2·4	115·4 ± 5·1	113·5 ± 2·9	145·7 ± 2·3	132·2 ± 2·2
Lactobacillus rhamnosus ATCC 53103	8·9 ± 0·7	9·5 ± 0·4	11·1 ± 0·4	13·9 ± 1·3	130·4 ± 3·5	137·4 ± 3·2
Lactobacillus rhamnosus ATCC 7469	10·7 ± 1·0	10·1 ± 0·9	25·2 ± 0·4	19·0 ± 0·8	108·2 ± 1·9	118·2 ± 3·1
Enterococcus faecium ATCC 19434	6·9 ± 0·2	5·8 ± 0·7	−3·3 ± 0·6	−3·5 ± 0·4	147·4 ± 1·4	124·6 ± 3·4
Lactobacillus salivarius HE2	10·2 ± 2·1	8·3 ± 1·0	17·9 ± 0·0	11·4 ± 0·6	184·3 ± 4·8	143·1 ± 1·6
Lactobacillus salivarius HC5	11·7 ± 1·0	11·2 ± 0·8	31·7 ± 1·7	22·2 ± 0·6	172·6 ± 3·8	136·8 ± 1·9
Lactobacillus salivarius CE4	9·5 ± 1·6	7·0 ± 4·2	14·3 ± 3·4	12·8 ± 0·8	57·2 ± 0·7	66·1 ± 1·02
Lactobacillus salivarius CE5	8·4 ± 1·1	8·8 ± 1·6	18·3 ± 0·8	16·3 ± 1·9	66·0 ± 0·4	73·7 ± 2·8
Lactobacillus salivarius CC1	6·6 ± 1·3	10·2 ± 0·8	3·9 ± 1·2	7·5 ± 0·3	92·0 ± 1·8	102·3 ± 3·6
Lactobacillus salivarius CC3	7·5 ± 0·7	7·7 ± 1·4	6·7 ± 0·6	12·0 ± 0·9	45·5 ± 1·0	56·9 ± 1·4
Lactobacillus salivarius CC5	10·3 ± 0·8	11·3 ± 2·4	8·0 ± 1·3	9·7 ± 0·4	48·6 ± 1·6	57·2 ± 2·3
Lactobacillus salivarius CC6	8·0 ± 0·8	6·2 ± 0·8	8·9 ± 0·2	10·5 ± 1·5	49·7 ± 1·1	59·0 ± 1·4
Lactobacillus crispatus HE6	8·9 ± 1·9	7·2 ± 1·6	74·3 ± 2·3	77·3 ± 2·4	141·1 ± 2·7	122·2 ± 0·3
Lactobacillus crispatus HC1	4·9 ± 1·4	5·6 ± 2·1	71·4 ± 1·4	73·8 ± 2·3	129·0 ± 0·9	113·3 ± 1·1
Lactobacillus crispatus CE3	5·7 ± 2·4	8·1 ± 2·3	6·8 ± 0·4	8·5 ± 0·7	45·2 ± 0·7	57·6 ± 0·6
Lactobacillus crispatus CC2	6·2 ± 1·7	8·8 ± 1·2	4·0 ± 1·6	8·8 ± 0·9	39·1 ± 1·9	56·9 ± 1·4
Lactobacillus crispatus CC4	6·3 ± 0·6	8·2 ± 2·1	36·5 ± 1·5	51·6 ± 1·3	83·1 ± 0·8	87·2 ± 2·8
Lactobacillus crispatus CC7	6·3 ± 0·6	7·8 ± 2·0	11·7 ± 1·1	6·8 ± 0·4	185·4 ± 2·6	142·3 ± 0·4
Lactobacillus kitasatonis HE1	5·4 ± 1·2	3·1 ± 1·7	113·5 ± 1·7	115·4 ± 2·7	132·0 ± 0·9	132·3 ± 5·1
Lactobacillus kitasatonis HE4	3·6 ± 1·2	4·7 ± 0·8	75·0 ± 1·3	78·3 ± 0·6	125·7 ± 2·0	115·1 ± 1·5
Lactobacillus ingluviei HC2	6·5 ± 0·5	6·7 ± 0·6	10·1 ± 0·8	28·3 ± 2·9	115·2 ± 2·1	106·4 ± 2·4
Lactobacillus ingluviei HC7	7·1 ± 0·5	8·5 ± 0·9	2·3 ± 0·2	4·0 ± 0·7	61·7 ± 2·2	67·0 ± 4·1
Lactobacillus reuteri HC3	9·3 ± 1·0	9·3 ± 2·8	3·6 ± 1·2	6·7 ± 1·3	104·5 ± 3·0	105·0 ± 2·7
Lactobacillus reuteri HC4	7·4 ± 2·1	9·2 ± 1·0	11·0 ± 0·8	7·6 ± 1·1	176·6 ± 1·1	143·1 ± 2·2
Lactobacillus vaginalis HE3	2·6 ± 1·5	2·2 ± 0·7	70·5 ± 1·2	71·4 ± 1·0	136·9 ± 2·8	120·0 ± 0·5
Lactobacillus gallinarum HE5	2·8 ± 1·0	2·8 ± 2·6	49·5 ± 0·2	61·6 ± 0·7	135·3 ± 2·5	119·2 ± 0·3
Lactobacillus oris HC6	5·7 ± 1·0	6·9 ± 1·8	10·3 ± 1·5	7·6 ± 1·7	193·8 ± 1·8	156·0 ± 2·3
Enterococcus faecium CE1	6·3 ± 1·1	7·2 ± 0·7	1·0 ± 1·2	0·1 ± 0·3	61·3 ± 1·6	69·5 ± 0·7
Lactobacillus plantarum LQ80	10·1 ± 0·4	9·7 ± 0·9	19·7 ± 0·9	18·0 ± 0·4	99·1 ± 4·2	111·9 ± 3·5

[1]Turbidity was determined before and after incubation. Difference of turbidity of control (*Salmonella* and MRS broth) was considered 100%; relative value was calculated in each LAB supernatant.
[2]*Salmonella* Enteritidis.
[3]*Salmonella* Typhimurium.
[4]Mean ± SD ($n = 3$).

instead of LAB supernatant. Turbidity was determined before and after the incubation, the difference of turbidity in the control culture was considered 100%, and the relative value was calculated in each LAB supernatant.

Production of short chain fatty acids

LAB strains were grown at 37°C for 48 h in modified MRS broth (standard MRS broth with 4% glucose). Bacterial cells were removed from the liquid culture by centrifugation (20,000 g, 10 min), and final pH was determined. The short-chain fatty acid concentration of the supernatant was analyzed by HPLC, equipped with a YMC-Pack FA column (250 × 6·0 mm; YMC Co., Ltd., Kyoyo, Japan). Concentrations of lactate, acetate and propionate were calculated using DL-methyl butyrate as an internal standard.

Table 2. *Short-chain fatty acid production of the LAB strains*

Strains	Acid production (mmol/L)			Final pH
	Lactate	Acetate	Propionate	
Lactobacillus delbrueckii JCM 1012	130·9 ± 6·1[1]	72·5 ± 4·3	7·0 ± 0·1	4·04 ± 0·26
Lactobacillus delbrueckii JCM 1248	171·5 ± 26·1	76·6 ± 1·6	3·6 ± 0·0	4·01 ± 0·01
Lactobacillus intestinalis JCM 7548	285·9 ± 46·6	90·0 ± 3·6	18·4 ± 4·3	3·83 ± 0·01
Lactobacillus amylovorus JCM 1126	357·6 ± 20·5	80·2 ± 1·9	19·4 ± 2·8	3·70 ± 0·01
Lactobacillus paracasei JCM 1181	294·3 ± 56·5	74·8 ± 1·4	10·5 ± 0·5	3·78 ± 0·11
Lactobacillus manihotivorans JCM 12514	308·8 ± 28·1	77·1 ± 2·6	10·1 ± 0·9	3·73 ± 0·01
Lactobacillus brevis JCM 1059	94·2 ± 1·2	94·9 ± 2·1	3·6 ± 0·0	4·50 ± 0·04
Lactobacillus johnsonii JCM 2012	310·0 ± 24·6	81·9 ± 8·5	3·8 ± 0·2	3·66 ± 0·03
Lactobacillus fermentum JCM 1173	227·6 ± 10·6	85·5 ± 2·4	3·6 ± 0·1	3·86 ± 0·01
Lactobacillus reuteri JCM 1112	208·0 ± 16·9	84·5 ± 3·1	3·6 ± 0·1	3·94 ± 0·00
Lactobacillus plantarum JCM 1149	294·7 ± 60·6	79·4 ± 10·1	3·7 ± 0·1	3·72 ± 0·01
Lactobacillus animalis JCM 5670	191·6 ± 5·4	74·5 ± 6·9	3·5 ± 0·1	3·92 ± 0·01
Lactobacillus gasseri JCM 1131	300·3 ± 15·1	83·4 ± 3·3	3·7 ± 0·1	3·73 ± 0·02
Lactobacillus paracasei JCM 8130	109·8 ± 10·2	69·0 ± 0·5	3·5 ± 0·0	4·27 ± 0·05
Lactobacillus casei JCM 1134	246·7 ± 16·6	74·7 ± 3·3	3·6 ± 0·1	3·79 ± 0·01
Lactobacillus rhamnosus ATCC 53103	250·8 ± 40·2	74·1 ± 3·1	10·1 ± 3·7	3·72 ± 0·04
Lactobacillus rhamnosus ATCC 7469	374·5 ± 20·5	75·5 ± 2·8	7·8 ± 0·2	3·60 ± 0·01
Enterococcus faecium ATCC 19434	106·5 ± 7·2	72·6 ± 4·4	3·6 ± 0·1	4·37 ± 0·01
Lactobacillus salivarius HE2	334·5 ± 34·4	92·0 ± 5·5	7·1 ± 5·0	3·79 ± 0·02
Lactobacillus salivarius HC5	1030·1 ± 88·6	228·9 ± 27·1	11·5 ± 4·5	3·72 ± 0·02
Lactobacillus salivarius CE4	212·1 ± 19·2	72·3 ± 0·6	7·4 ± 1·3	3·85 ± 0·01
Lactobacillus salivarius CE5	225·3 ± 14·6	73·5 ± 2·3	6·6 ± 0·9	3·81 ± 0·01
Lactobacillus salivarius CC1	228·3 ± 17·0	73·7 ± 2·2	6·0 ± 0·0	3·82 ± 0·01
Lactobacillus salivarius CC3	221·3 ± 14·0	73·7 ± 1·6	6·9 ± 0·8	3·83 ± 0·00
Lactobacillus salivarius CC5	217·7 ± 7·0	72·5 ± 0·3	6·9 ± 0·8	3·83 ± 0·01
Lactobacillus salivarius CC6	220·4 ± 13·8	71·9 ± 1·8	9·4 ± 0·3	3·83 ± 0·00
Lactobacillus crispatus HE6	1219·8 ± 9·7	351·9 ± 9·0	8·5 ± 4·0	3·88 ± 0·00
Lactobacillus crispatus HC1	1236·5 ± 39·0	326·8 ± 14·4	9·8 ± 0·5	3·82 ± 0·01
Lactobacillus crispatus CE3	223·5 ± 6·9	81·6 ± 1·3	6·1 ± 0·1	3·80 ± 0·01
Lactobacillus crispatus CC2	222·4 ± 7·0	84·3 ± 2·6	6·0 ± 0·0	3·81 ± 0·02
Lactobacillus crispatus CC4	129·6 ± 8·2	76·0 ± 1·7	6·0 ± 0·0	4·17 ± 0·01
Lactobacillus crispatus CC7	212·5 ± 4·6	80·5 ± 1·0	6·4 ± 0·6	3·83 ± 0·01
Lactobacillus kitasatonis HE1	478·5 ± 90·3	126·3 ± 23·8	20·3 ± 11·9	3·77 ± 0·01
Lactobacillus kitasatonis HE4	199·2 ± 15·6	91·4 ± 3·1	5·0 ± 0·3	4·15 ± 0·02
Lactobacillus ingluviei HC2	1684·1 ± 92·1	563·7 ± 29·9	9·9 ± 2·3	3·90 ± 0·01
Lactobacillus ingluviei HC7	308·2 ± 11·4	105·4 ± 5·9	4·3 ± 0·3	3·90 ± 0·01
Lactobacillus reuteri HC3	1130·5 ± 138·2	484·3 ± 24·5	5·9 ± 2·9	3·98 ± 0·01
Lactobacillus reuteri HC4	1042·6 ± 164·4	517·5 ± 18·9	7·5 ± 1·9	4·19 ± 0·00
Lactobacillus vaginalis HE3	83·3 ± 1·9	97·1 ± 3·4	3·9 ± 0·3	4·67 ± 0·01
Lactobacillus gallinarum HE5	1168·2 ± 110·9	307·7 ± 29·0	15·9 ± 14·9	3·84 ± 0·02
Lactobacillus oris HC6	468·0 ± 360·8	217·7 ± 163·9	6·9 ± 4·5	4·06 ± 0·01
Enterococcus faecium CE1	103·9 ± 11·3	69·0 ± 2·9	6·1 ± 0·1	4·34 ± 0·01
Lactobacillus plantarum LQ80	263·7 ± 13·9	76·6 ± 1·6	6·1 ± 0·1	3·72 ± 0·01

[1]Mean ± SD ($n = 3$)

Survival and growth at low pH and in the presence of bile salts in isolated LAB

The assay for acid tolerance was performed as follows. Test tubes containing 3 ml acidified MRS (pH 2·0 with HCl) were each inoculated with 333 µl of freshly grown test bacteria (grown at 37°C for overnight in MRS broth). Survival was analyzed by plating ten-fold dilutions onto MRS agar before and after 1 h of incubation at 37°C. In the bile tolerance assay, test tubes containing 3 ml of MRS, or MRS containing 0·3% oxgall (Sigma-Aldrich, St Louis, MO, USA), were each inoculated with 30 µl of the liquid culture (grown at 37°C for overnight in MRS broth), and incubated for 16 h. Survival was analyzed by plating of ten-fold dilutions onto MRS agar.

RESULTS

The size of the inhibition zone differed slightly between *S.* Enteritidis and Typhimurium; however, the mean size of the clear zone differed strongly among the LAB species (Table 1). Most *L. salivarius* strains showed larger inhibition zones, and the results of the co-incubation assay of supernatant of each LAB strain and *Salmonella* also showed that these strains could inhibit *Salmonella* growth effectively. *L. plantarum* (JCM

1149 and LQ80), *L. rhamnosus* ATCC 53103 and *L. rhamnosus* ATCC 7469 exhibited a powerful inhibitory effect on each *Salmonella* strain. The supernatants of these strains showed higher acid production and lower final pH (Table 2). However, neutralized supernatants of these strains did not inhibit the growth of Salmonella.

The results of the acid tolerance test showed that all *L. salivarius, L. kitasatonis* strains, and one of the *L. ingluviei* could not survive in a low pH (2·0) environment after 1 h incubation (Table 3). In the bile acid tolerance assay, only *L. kitasatonis* HE4 was able to grow in MRS containing 0·3% oxgall, whereas the growth of the other strains was inhibited (Figure). A large inhibition was observed in most *L. salivarius* and *L. crispatus* strains; however, other strains had moderate tolerance against bile salts.

Table 3. *Survival at low pH of the LAB strains (log CFU/ml)*

Strain	Before	After
Lactobacillus salivarius HE2	7·47 ± 0·31	–
Lactobacillus salivarius HC5	7·02 ± 0·08	–
Lactobacillus salivarius CE4	7·28 ± 0·06	–
Lactobacillus salivarius CE5	7·45 ± 0·05	–
Lactobacillus salivarius CC1	7·49 ± 0·06	–
Lactobacillus salivarius CC3	7·29 ± 0·03	–
Lactobacillus salivarius CC5	7·46 ± 0·06	–
Lactobacillus salivarius CC6	7·64 ± 0·04	–
Lactobacillus crispatus HE6	7·78 ± 0·09	–
Lactobacillus crispatus HC1	7·83 ± 0·06	–
Lactobacillus crispatus CE3	7·47 ± 0·03	5·48 ± 0·74
Lactobacillus crispatus CC2	7·45 ± 0·10	–
Lactobacillus crispatus CC4	7·79 ± 0·10	5·11 ± 0·16
Lactobacillus crispatus CC7	7·41 ± 0·12	5·02 ± 0·03
Lactobacillus kitasatonis HE1	7·76 ± 0·07	–
Lactobacillus kitasatonis HE4	7·01 ± 0·15	–
Lactobacillus ingluviei HC2	8·10 ± 0·05	5·39 ± 0·95
Lactobacillus ingluviei HC7	7·84 ± 0·02	–
Lactobacillus reuteri HC3	8·03 ± 0·07	7·42 ± 0·03
Lactobacillus reuteri HC4	7·84 ± 0·06	7·33 ± 0·06
Lactobacillus vaginalis HE3	7·51 ± 0·10	7·49 ± 0·03
Lactobacillus gallinarum HE5	7·43 ± 0·05	–
Lactobacillus oris HC6	7·90 ± 0·02	7·83 ± 0·17
Enterococcus faecium CE1	7·42 ± 0·19	6·38 ± 0·13
Lactobacillus plantarum LQ80	7·95 ± 0·05	5·30 ± 0·30
Lactobacillus rhamnosus ATCC 53103	7·78 ± 0·05	5·63 ± 0·13

Survivability was analyzed by plating of ten-fold dilutions onto MRS agar before and after 1 h of incubation at 37°C in MRS (pH 2.0).
"-" was below 5.0 log CFU/ml.
1 Mean±SD (n=3)

DISCUSSION

As an initial screening for antimicrobial properties of each strain, we tested inhibitory activity against *S.* Enteritidis and Typhimurium, and a larger inhibition zone was observed in several *L. salivarius* strains. This result agrees with the report of Casey *et al.* (2004), who observed that several *L. salvarius* strains isolated from swine caecal and faecal samples inhibited the growth of pathogenic microorganisms, including *Salmonella*. Van Coillie *et al.* (2007) made use of the same method to evaluate the inhibitory activity of LAB strains against *Salmonella*. They observed a decrease of pH under the paper disks after incubation of the LAB strains, and a smaller radius of inhibition zones on the buffered MRS plates compared to non-buffered plates. Thus, the inhibitory effect was probably the result of organic acid production. *L. salivarius* CC3, CC5, CC6, *L. crispatus* CC2 and CE3 exert medium or large inhibition zones and lower turbidity in co-incubation of the intact culture supernatant and *Salmonella*, whereas co-incubation with pH-neutralised culture supernatant also showed lower turbidity compared to other LAB. It was previously reported that some LAB strains have antagonistic activity against gram-negative pathogens, producing a bactericidal substance (bacteriocin) that is neither lactic acid nor hydrogen peroxide (Edens *et al.*, 1997). In this experiment, whereas we did not confirm the existence of bacteriocin, our results show that these strains may have secreted this non-lactic acid antibacterial molecule.

In order to function efficiently as an antimicrobial agent in the lower sections of the intestinal tract, orally delivered bacteria have to survive through the gastrointestinal tract to their site of function. The first major barrier to overcome during this passage is survival in the acidic environment of the proventriculus and gizzard. Van Coillie *et al.* (2007) isolated *Lactobacillus* strains from hens, and about 50 representative strains were evaluated for acid tolerance with incubating acidic MRS broth (pH 3·0). Whereas the estimating method and criteria were different from our experiment, no bacterial growth was observed, although half of the strains could survive in that environment.

If these bacteria survive through the gastric environment, the next challenge is to withstand the presence of bile acids, a major hindrance to bacterial survival in the small intestine. Among the strains tested for survival at low pH and in the presence of bile salts, *L. rhamnosus* ATCC 53103 (GG) exhibited a higher survival potential than other tested LAB. Jacobsen *et al.* (1999) reported that this strain survived better in the gastrointestinal tract than other strains in a human *in vivo* trial. Our results are in agreement with those reported by Fayol-Messaoudi *et al.* (2005) showing that cell-free culture supernatant of *L. rhamnosus* GG has antagonistic activity against *S.* Typhimurium. The effect of bile salts on the survival of *lactobacilli* has been investigated, and a strong correlation of the bile salt hydrolase

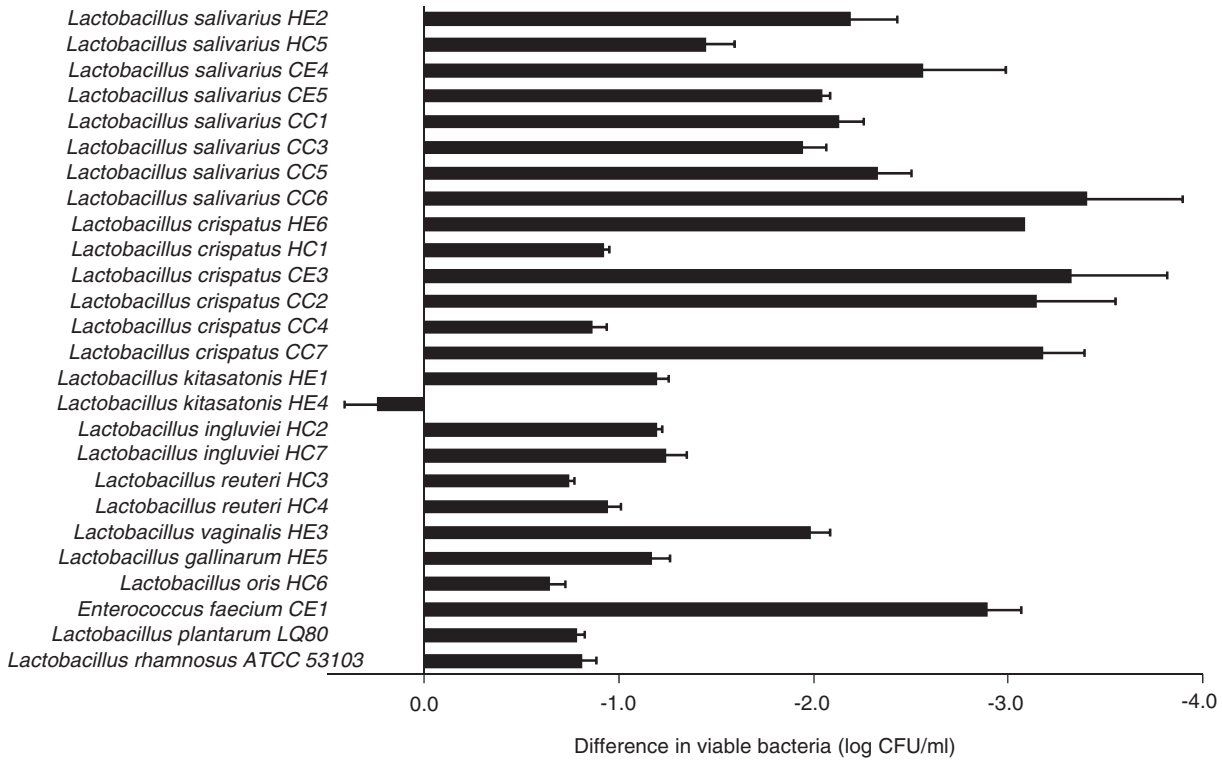

Figure. *Effect of Oxgall supplementation on survivability of LAB strains. Survivability was analyzed by incubating at 37°C for 16 h in MRS with or without 0·3% Oxgall. Difference of viable bacteria = CFU of MRS with 0·3% Oxgall - MRS without oxgall. Bars indicate the SD of the mean* (n = *3).*

(BSH) activity of *lactobacilli* with cholic acid accumulation and growth inhibition was reported (Tannock *et al.*, 1997). Therefore, the different BSH activity can also be considered as a reason for the different inhibition rates among the strains used in this experiment, although BSH activity was not determined here. Van Coillie *et al.* (2007) reported that, in the low *in vitro* tolerance against bile salts, only a slightly lower caecal colonization capacity was observed for the *Lactobacillus* strain when compared with the strains that had high resistance to bile salts. Resistance to bile salts is a prerequisite for beneficial microorganisms, and it is controversial as to whether this *in vitro* bile acid tolerance test is suitable for selecting effective strains or not.

The results obtained in the present study demonstrate the ability of some poultry LAB isolates to both inhibit the growth of *Salmonella* and survive passage through the gastrointestinal tract. Some of the isolates have powerful potential for the inhibition of *Salmonella* growth in the poultry digestive tract. Based on this *in vitro* result, evaluations *in vivo* for colonization and inhibition of *Salmonella* infection are necessary.

ACKNOWLEDGEMENTS

We would like to thank Dr Kiyoshi Tajima for sequencing the 16S rRNA gene of LAB, and Dr Masaru Nomura, Dr Hiromi Kimoto-Nira and Dr Miho Kobayashi for their valuable advice in this study. This study was supported by a grant-in-aid from the Research Project for Ensuring Food Safety from Farm to Table LP-5104, of the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**: 3389–3402.

Casey, P.G., Casey, G.D., Gardiner, G.E., Tangney, M., Stanton, C., Ross, R.P., Hill, C. & Fitzgerald, G.F. (2004) Isolation and characterization of anti-Salmonella lactic acid bacteria from the porcine gastrointestinal tract. *Letters in Applied Microbiology*, **39**: 431–438.

Edens, F.W., Parkhurst, C.R., Casas, I.A. & Dobrogosz, W.J. (1997) Principles of *Ex Ovo* competitive exclusion and *In Ovo* administration of *Lactobacillus reuteri*. *Poultry Science*, **76**: 179–196.

Fayol-Messaoudi, D., Berger, C.N., Coconnier-Polter, M., Moal, V.L. & Servin, A.L. (2005) pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enteric* serovar Typhimurium. *Applied and Environmental Microbiology*, **71**: 6008–6013.

Gusils, C., Pérez Chaia, A., González, S. & Oliver, G. (1999) Lactobacilli isolated from chicken intestines: potential use as probiotics. *Journal of Food Protection*, **62**: 252–256.

Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayhord, A.E., Møller, P.L., Michaelsen, K.F., Pærregaard, A., Sandström, B., Tvede, M. & Jakobsen, M. (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology*, **65**: 4949–4956.

Jin, L.Z., Ho, Y.W., Abdullah, N., Ali, M.A. & Jalaludin, S. (1996) Antagonistic effects of intestinal lactobacillus isolates on pathogens of chicken. *Letters in Applied Microbiology*, **23**: 67–71.

Johnson, J.L. (1991) Isolation and purification of nucleic acids, in: Stackebrandt, E. & Goodfellow, M. (Eds) *Nucleic Acid Techniques in Bacterial Systematics*, pp. 1–19 (New York, Wiley).

Lane, D.J. (1991) 16S/23S rRNA sequencing, in: Stackebrandt, E. & Goodfellow, M. (Eds) *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175 (New York, Wiley).

Ministry of Health, Labour and Welfare (2009) Information on food poisoning in Japan. Available: http://www.mhlw.go.jp/topics/syokuchu/index.html.

Nurmi, E. & Rantala, M. (1973) New aspects of salmonella infection in broiler production. *Nature*, **241**: 210–211.

Oyarzabal, O.A. & Conner, D.E. (1995) In vitro fructooligosaccharide utilization and inhibition of salmonella spp. by selected bacteria. *Poultry Science*, **74**: 1418–1425.

Tannock, G.W., Bateup, J.M. & Jenkinson, H.F. (1997) Effect of sodium taurocholate on the in vitro growth of lactobacilli. *Microbial Ecology*, **33**: 163–167.

Van Coillie, E., Goris, J., Cleenwerck, I., Grijspeerdt, K., Botteldoorn, N., Van Immerseel, F., De Buck, J., Vancanneyt, M., Swings, J., Herman, L. & Heyndrickx, M. (2007) Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella enteritidis*. *Journal of Applied Microbiology*, **102**: 1095–1106.