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Full Length Research Paper

Screening of lactobacilli bacteria isolated from gastrointestinal tract of broiler chickens for their use as probiotic

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A study was conducted to isolate lactobacilli from the gastrointestinal (GI) tract of broiler chickens and to evaluate their application as a probiotic supplement. The GI contents of 12 broiler chickens from three different ages were collected and cultured on Man Rogosa and Sharp (MRS) agar. In order to determine bacterial species, different grown colonies were separated and subjected to the sequencing of the 16s ribosomal DNA fragment. An auto-aggregation test was performed for 39 isolated lactobacilli bacteria. Eight species were selected because of their convenient aggregation. *In vitro* tests including antibacterial activity, resistance to low pH, resistance to bile extract, ability to produce H_2O_2, cell surface hydrophobicity and adhesion activities on crop epithelial cells were performed for evaluating of probiotic potential for each strain. These tests were ranked according to their priority for probiotic strains. The total score obtained from different experiments is indicator of suitable strain. Under the *in vitro* conditions and with respects to the probiotic traits, *Lactobacillus salivarius* caeca4 and *Lactobacillus crispatus* caeca10 were suggested as probiotic strain, and can be evaluated in an *in vivo* conditions of commercial birds.

Key words: Lactobacilli, probiotic, gastrointestinal tract, chicken.

INTRODUCTION

Lactobacilli are members of the lactic acid bacteria group, a broadly family of microorganism spectrum of bacteria that ferment various hexoses to lactic acid. They are low G+C, Gram positive and catalase negative. These bacteria colonize in the small intestine and caeca of chickens, a week after hatch (Mead, 1997). They help maintain the natural balance of organisms (microflora) in the intestines and help support a healthy digestive system (Dunne et al., 1999). The use of lactobacilli regards to the following reasons is on focus: 1- lactobacilli exhibit “competitive exclusion”, a property that inhabits the growth of pathogen bacteria. 2- lactobacilli conveniently attach to the epithelial cells of the intestine and 3- lactobacilli are known as safe symbiosis organisms for the host and enhance the immune system function.

It was found that the consumption of probiotic shows

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beneficial results in farm animals. Probiotics have the potential of preventing the growth of intestinal pathogen bacteria, specially *salmonella*, in chickens (Pascual et al., 1999). Kizerwetter-Swida and Binek (2009) demonstrated that the *Lactobacillus salivarus* 3d strain reduced the number of *Salmonella enteritidis* and *Clostridium perfringens* in the treated chickens.

Lan et al. (2004) showed that lactobacilli bacteria, when used as a pro-biotic, preserve the balance and maintain natural stability of microflora in the intestines of chickens following heat stress. Some experiments have indicated that microbial supplements may be use as an alternative for antibiotics in prevention dose. In this way, it has been observed that lactobacilli bacteria are effective on the growth of chicks same as the antibiotics (Pandey et al., 2000, Murry et al., 2004b, Kalavathy et al., 2008). It has been pointed out that probiotics developed the productive performance in poultry (Russell and Grimes, 2009) and reduced the body and serum fat content (Kalavathy et al., 2006, 2008)

Many probiotics have been introduced by screening the natural intestinal microflora of farm animals. Determination of an optimal species for probiotic usage is completely empirical (Ehrmann et al., 2002) and some criteria must be considered for selection. The important criteria consist of functional characteristics such as the resistance to environmental conditions of the digestive tract, adhesiveness of lactobacilli to the epithelial cells, and production of preventive substances against the pathogenic bacteria. The last property is considered as an important ecological factor that determine dominant bacteria in some ecosystems like the intestine (Busarcevic et al., 2008). The selected bacterial strains must be genetically stable and exhibit an adequate growth rate both *in vivo* and *in vitro* conditions. Furthermore, the bacteria must have a high viability during processing and storage. This study was conducted in order to isolate the native lactobacilli from different parts of the GI tract of the broiler chickens for evaluating of their probiotic potential.

MATERIALS AND METHODS

Isolation of bacteria

Bacteria were isolated from GI tract of 12 Ross broiler chickens from commercial poultry houses (Placed in Mashhad, Iran) at three different ages (15, 30 and 40 days old). They were fed based on standard diets without antibiotic supplement. The contents of the crop, proventriculus, gizzard, small intestine and caeca were separately removed under sterile conditions. Samples from each site were serially diluted in normal saline (from 0.1 to 0.001), plated onto Man Rogosa and Sharp (MRS) medium and incubated anaerobically at 37°C for 36 h. The isolated pure cultures were evaluated by catalase test, Gram stain and bacterial morphology. The bacterial samples were stored at 4°C for a short time and lyophilized in 15% sucrose for long-term storage.

Lactobacillus genus identification by specific genus primers

DNA was extracted from the Gram positive bacteria as described by Hilmi et al. (2007). A 203-bp fragment at nucleotides 40 to 243 of 16S ribosomal region were amplified by specific genus primers.

Forward primer included: 5'-CTT GTA CAC ACC GCC CGT CA-3' and reverse primer included: 5'-CTC AAA ACT AAA CAA AGT TTC-3'. The polymerase chain reaction (PCR) was started with heating at 94° C for 3 min followed by 35 cycles of PCR (consisting of 30 s at 92°C, 30 s at 55°C, 60 s at 72°C) and a final cycle 5 min at 73°C. The PCR products were analyzed on 1% agarose gel.

Sequencing genes in the 16S ribosomal region

For sequencing genes in the 16S rDNA region, DNA from *Lactobacillus* genus bacteria were amplified by universal primers pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pE' (5'-CCG TCA ATT CCT TTG AGT TT-3') (Edwards et al., 1989). The primers hybridized to the 16S rDNA gene at nucleotides 8 - 28 and 928 - 908 in *Escherichia coli*. The PCR was followed by heating at 95°C for 2 min and 35 cycles (consisting of 95°C for 45 s, 53°C for 45 s and 72°C for 60 s) and a final cycle 73°C for 3 min. After the electrophoresis on agarose gel, the gel containing the desired PCR products was cut out and extracted using a gel extraction kit (Bioneer, Daejeon 306-220, Korea). The DNA fragments were sequenced by the Seqlab Co. (Seqlab, Göttingen, Germany) and sequences were compared with available sequences in GenBank using the BLASTN tool through the National Center for Biotechnology Information (NCBI) server (http://blast.ncbi.nlm.nih.gov/Blast/, 2010). Sequence homologies of more than 97% regarded as belonging to the same species (Tannock, 1999).

Auto-aggregation test

This test was performed according to Reniero et al. (1992). The auto-aggregation phenotype was monitored for each bacterium within 2 h. Auto-aggregation was considered positively when suspended cells gravitated in bottom of tube and left a clear supernatant.

Detection of antimicrobial activity

Antimicrobial activity of selected bacteria was evaluated in presence of *Salmonella typhimurium* and *E. coli*. The experiment was performed by well diffusion assay (Schillinger and Lucke, 1989). The pH of supernatant fluid was measured to examine the correlation between inhibitory activity and acidity of supernatant. Inhibitory zones around the wells were screened for each strain after overnight at 37°C. The experiment was carried out three times and data were displayed as the mean of radius of inhibitory zone.

Tolerance to acidic pH

The evaluation of the bacteria in an acidic environment were performed by incubating them from one-half to 4 h in various pHs. Lactobacilli bacteria were cultured anaerobically in MRS broth at 37°C for 24 h and refreshed in 10 ml MRS broth for another 24 h. The media tubes containing bacteria were centrifuged at 1700 × g for 15 min at 4°C and settled bacteria washed twice with a

phosphate buffered saline (PBS). The washed samples were diluted 1/20 in PBS, pH= 2 ,3 ,4 ,and 5 and were incubated for 0.5, 1, 2, 3 and 4 h at 37°C. The samples were plated after an appropriate dilution, on MRS agar. The enumeration of surviving cells were carried out following 48 h incubation at 37°C overnight.

Growth in present of taurocholic acid and ox bile

In this test, growth of lactobacilli in presence of different dilution of taurocholic acid (Fluka, sigma-Aldrich GmbH, Buchs; cat 86339) and ox bile was assessed (Fluka, Sigma-Aldrich GmbH, Buchs; cat.70168). A volume of 20 µl of bacteria were cultured in MRS broth containing 0.0, 2 and 0.3% ox bile. For evaluation of growth in presence of taurocholic acid, the bacteria were cultured in MRS broth containing 0.0, 7, 14, 21 mmol l^{-1} sodium taurocholate. The optical densities (O.D) were monitored during 24 h with 1 h interval. Experiments were executed in four replications within 96 well plate.

Hydrophobicity and adherence

Microbial surface hydrophobicity was evaluated by the adherence to the non- polar solvents. Two solvents were used according to Pelletier et al. (1997). Briefly, lactobacillus cultures, in stationary phase, were centrifuged and washed twice with PBS and their absorbance adjusted to 0.6 at 600 nm (A_0). A volume of 1 ml of xylene (Merk Schuchardt OHG, Hohenbrunn, Germany; cat.818754) or n-hexadecan (Merk Schuchardt OHG, Hohenbrunn, Germany; cat.8206330250) was added to 2 ml of adjusted cell suspension. After 10 min incubation at room temperature, suspension was stirred vigorously for 2 min. The aqueous phase was separated, incubated for 30 min and its absorbance was measured at 600 nm (A_1). The percentage of hydrophobicity was calculated as (1-A1/A0) ×100.

The selected bacteria were also evaluated for adherence to crop epithelial cells. This test was performed as described by Fuller (1973) with a few modifications. Briefly internal surface of the crop from 25-days old chickens were gently scraped off by the edge of microscope slide. The scraped epithelial cells were then suspended in PBS and adjusted to 8×10^5 cfu. The bacterial cells concentration was also adjusted to 1.5×10^8 cfu ml^{-1} based on the McFarland standard. A volume of 100 µl of bacterial suspension was added to 400 µl of the epithelial cells suspension and incubated under shaking at 37°C for 30 min. A 10 µl of sample was mixed with equal volume of trypan blue dye and attachment of bacteria to epithelial cells were observed by light microscope.

Hydrogen peroxide production

Hydrogen peroxide production was evaluated by culturing the bacteria in adjusted media, as described by Eschenbach et al. (1989). Briefly, 15 µl refreshed MRS broth containing lactobacilli were inoculated in 10 ml MRS agar containing 2.5 mg 3,39,5,59-tetramethylbenzidine (TMB); (Sigma-Aldrich GmbH, Buchs; cat. T2885) and 0.1 mg horseradish peroxidase (Boehringer-mannheiman GmbH; cat.83422430). In this mixture, colonies that are able to produce hydrogen peroxide develop a color of blue. The plates were incubated in 37°C under 5% CO_2 for 24 h and finally were exposed to air for 30 min.

Statistical method

All quantitative data were subjected to ANOVA analysis (SASInstitute and 2004) appropriate for completely randomized design. Treatment means were compared using Duncan's multiple rang test when level of significance was more than 0.05. The experimental protocols were reviewed and approved by the Animal Care Committee of Ferdowsi University of Mashhad, Iran.

RESULTS

Determination of the lactobacilli species

The isolated bacteria which presented the specifications of *Lactobacillus,* such as positive Gram stain, catalase negative reaction, and bacillus shape were subjected to further analysis. Amplification of the 16S rDNA fragment (0.24 kbp) using specific genus primers assured that all isolated bacteria belong to the *Lactobacillus* genus (Figure 1). The sequencing of the 16S rDNA fragment (0.9 kbp) determined the lactobacilli at the species level. Based on sequencing result, three species from proventriculus and gizzard, four species from small intestine and caeca were isolated (Table 1). The similar strains were identified through their phylogenic relationship and recognized as same species (Figure 2).

Auto-aggregation test

Auto-aggregation time was screened in 39 colonies of lactobacilli isolated from the GI tract of broiler chickens. For screening, the total time for significant aggregation was up to 120 min. The samples that aggregated in less than 90 min were chosen for the next experiments. Eight bacteria that were selected in this step including*:* *Lactobacillus crispatus* c2, *Lactobacillus salivarus* c4 ,and *Lactobacillus crispatus* c8 from the crop, *Lactobacillus johansonii* g4 and *Lactobacillus crispatus* int8 from the proventriculus and intestine, respectively, *Lactobacillus reuteri* caeca 4, *Lactobacillus salivarus* caeca6*,* and *Lactobacillus crispatus* caeca10 from the cecea.

Inhibitory activity

The colonies of lactobacilli exhibited antibacterial activity against *E. coli* except one strain. *L. salivarius* c4 and *L. crispatus* caeca10 showed the most inhibitory activity ($p<0.05$) (Table 2). Acidity of bacterial supernatant was effective on inhibition of *E. coli* ($p<0.05$), whereas it had no effect on *salmonella* inhibition. *L. salivarius* c4 was the only strain that showed more inhibition than those of other strains on *salmonella* ($p<0.05$).

Tolerance to different acidic pH

All species survived in pH=4 and 5 and no difference in

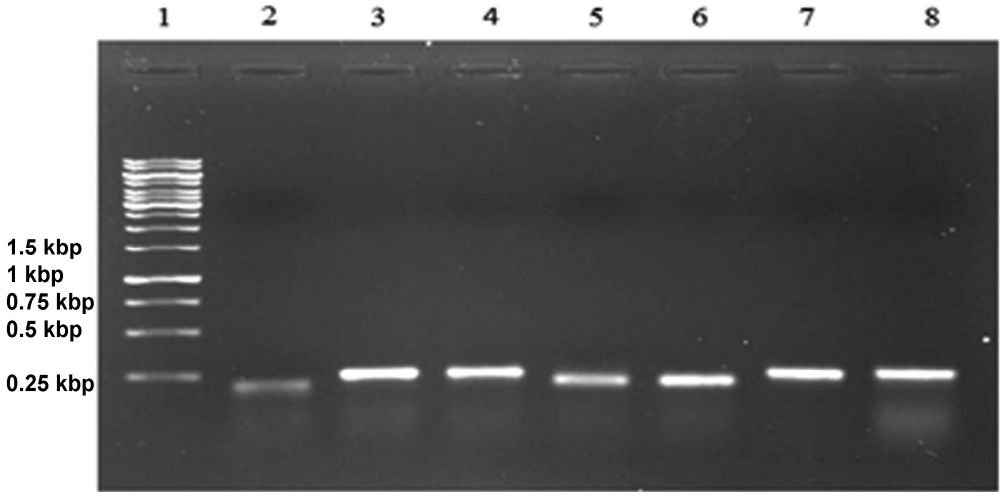

Figure 1. PCR products by specific genus primer. Lane 1: ladder (1 kbp), lane 2: *Lactobacillus reuteri* c1, lane 3: *Lactobacillus crispatus* c3, lane 4: *Lactobacillus crispatus* c5, lane 5: *Lactobacillus agillis* c6, lane 6: *Lactobacillus reuteri* c7, lane 7: *Lactobacillus crispatus* c8, lane 8: *Lactobacillus johansonii* c10.

Table 1. Isolated lactobacilli from GI tract of broiler chickens.

Species	Bacterial no. [1]	Similarity (%)	GenBank no.
Crop			
L. reuteri	c1	100	EU722746.1
L. crispatus	c2	100	Y17362.1
L. crispatus	c3	100	Y17362.1
L. salivarius	c4	100	DQ444477.1
L. crispatus	c5	100	Y17362.1
L. agilis	c6	98	M55803.1
L. reuteri	c7	99	EU722746.1
L. crispatus	c8	100	Y17362.1
L. reuteri	c9	100	EU722746.1
L. johnsonii	c10	100	EF187257.2
L. oris	c11	99	X9423.1
L. crispatus	c12	100	Y17362.1
L. crispatus	c13	100	Y17362.1
Proventriculus and gizzard			
L. crispatus	g1	100	Y17362.1
L. crispatus	g2	100	Y17362.1
L. salivarius	g3	100	DQ444477.1
L. johnsonii	g4	99	EF187257.2
L. salivarius	g5	100	DQ444477.1
L. crispatus	g6	100	Y17362.1
L. crispatus	g7	100	Y17362.1
L. johnsonii	g8	100	EF187257.2
Small intestine			
L. crispatus	int1	100	Y17362.1
L. crispatus	int2	100	Y17362.1

Table 1. Contd.

L. salivarius	int3	100	DQ444477.1
L. reuteri	int4	78	EU722746.1
L. johnsonii	int5	98	EF187257.2
L. crispatus	int7	100	Y17362.1
L. crispatus	int8	99	Y17362.1
Caeca			
L. sp. Autruche 5	caeca1	100	DQ448553.1
L. reuteri	caeca2	98	EU722746.1
L. crispatus	caeca3	99	Y17362.1
L. reuteri	caeca4	98	EU722746.1
L. crispatus	caeca5	99	Y17362.1
L. salivarius	caeca6	100	DQ444477.1
L. crispatus	caeca7	100	Y17362.1
L. salivarius	caeca8	100	DQ444477.1
L. salivarius	caeca9	98	DQ444477.1
L. crispatus	caeca10	99	Y17362.1

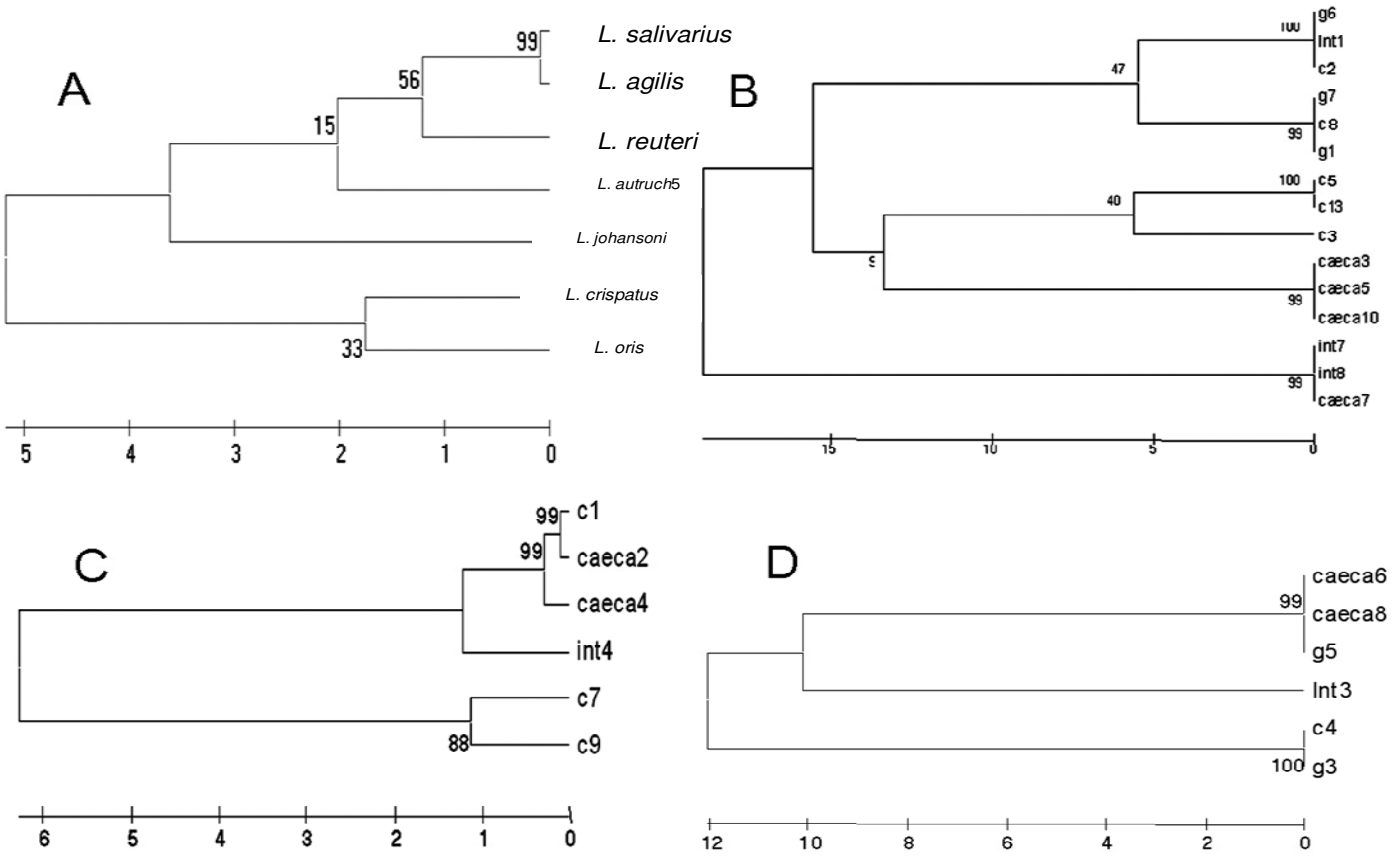

Figure 2. Dendrograph of phylogeny relationships in identified lactobacilli in this study with attention to their sequence of genes. Evolutions were accomplished by UPGMA method. The tree is drawn to scale and length each branch show phylogenic distance based on difference between repaced bases in each site. The evolutionary distances were computed using the Maximum Composite Likelihood Method. A) Phylogeny relationship between species; B) intra-species Phylogeny relationship in *L. crispatus;* C) intra-species Phylogeny relationship in *L. reuteri; D)* intra-species Phylogeny relationship in *L. salivarius.*

Table 2. Inhibitory activity of lactobacilli on *Salmonella* and *E. coli*.

Strain	Supernatant pH	Radius of Inhibitory zone*	
		Salmonella	***E. coli***
L. crispatus c2	3.93	0.167[b]	0.833 [b]
L. salivarus c4	3.95	2.00[a]	2.166[a]
L. crispatus c8	3.84	0.167 [b]	0.500 [b]
L. johnsonii g4	4.65	0.167 [b]	0.00 [b]
L. crispatus int8	3.86	0.167 [b]	0.833 [b]
L. reuteri caeca4	4.32	0.00 [b]	0.58 [b]
L. salivarus caeca6	4.37	0.167 [b]	0.333 [b]
L. crispatus caeca10	4.03	0.667 [b]	2.00[a]

*Means within a column lacking a common superscript differ ($p<0.05$).

Table 3. Percent of live lactobacilli after incubating at acidic pHs in different times.

Strain	pH 2				pH 3			
	0.5 h	**1 h**	**2 h**	**3 h**	**1 h**	**2 h**	**3 h**	**4 h**
L. crispatus c2	9.13	N.D	N.D	N.D	9.81	9.62	6.96	3.55
L. salivarus c4	0.00	N.D	N.D	N.D	10	5.16	6.42	4
L. crispatus c8	10.83	N.D	N.D	N.D	39.2	21.2	16.04	18
L. johnsonii g4	0.21	N.D	N.D	N.D	5.74	0.35	0.12	0.15
L. crispatus int8	0.28	N.D	N.D	N.D	30	18	10	3.79
L. reuteri cecu4	22.56	23.59	28.72	0.65	60.71	57.14	27.14	32.86
L. salivarus cecu6	0.18	N.D	N.D	N.D	40.28	44.44	16.67	15.28
L. crispatus cecu10	8.53	N.D	N.D	N.D	42	32.67	20.33	19.33

N.D = Not determined.

count for the allotted times was detected. Differences between species were observed in pH=3 and lower. The isolated lactobacilli from the cecae and *L. crispatus* c8 survived satisfactorily in pH=3. All lactobacilli tolerated pH=2 only at 0.5 h incubation, except *L. reuteri* caeca4. This bacterium survived after a maximum of 3 h incubation. *L. crispatus* c8 had an acceptable viability after *L. reuteri* caeca 4 in pH=2 at 0.5 h incubation (Table 3).

Effect of ox bile and taurocholic acid on growth

Growth of all of the bacteria decreased in presence of bile extract, but the rate of decrease was various among the species. *L. salivarius* c4 had the lowest sensitivity to 2% bile extract in the first 4 h. *L. crispatus* c8 displayed the most optical density (O.D) in comparison with other strains after 24 h incubation in 2% ox bile (Figure 3) but it had no difference with the optical density of *L. salivarius* c4 significantly. The growth of *L. crispatus* c8 increased in 0.3% ox bile (Figure 4). As shown in the Figures 3 and 4, isolated species from the cecae exhibited the lowest resistance to both ox bile concentrations. The lactobacilli species selected in this study were proliferated in the presence of taurocholic acid. *L. reuteri* caeca4 and *L. crispatus* c8 exhibited more growth than the other species in all of the concentration of taurocholic acid ($p<0.05$) (Figure 5).

Hydrophobicity and adherence

Hydrophobicity shows the properties of the outer membrane of bacteria in a hydrophilic environment. In the present study, this criterion was evaluated photo-metrically. There was no difference between bacteria in hydrophobicity property in each solvent ($p>0.05$). Values higher than 93% and between 66 to 93% were considered as strong hydrophobicity and hydrophobic, respectively (Ehrmann et al., 2002). Based on this pattern, *L. crispatus* caeca8 and *L. reuteri* caeca4 were strongly hydrophobic in xylen solution. *L. johnsonii* g4 was the only strain that showed strong hydrophobicity

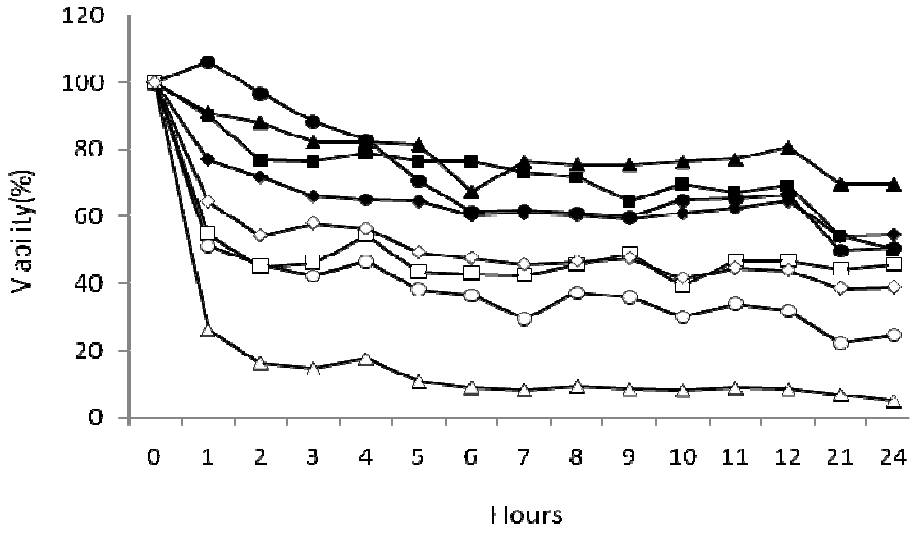

Figure 3. Survival of lactobacilli in presence of 2% ox bile extract. *L. crispatus* c2 (♦),*L. salivarus* c4 (●), *L. crispatus* c8 (▲), *L. johnsonii* g4 (■), *L. crispatus* int8 (Δ), *L. reuteri* caeca4 (○), *L. salivarus* caeca6(□), *L. crispatus* caeca10 (◊)

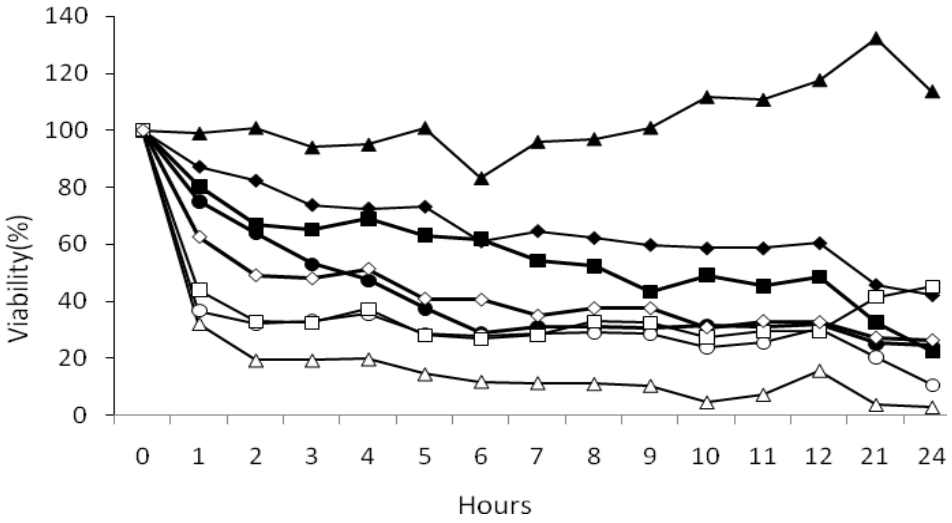

Figure 4. Survival of lactobacilli in presence of 0.3% ox bile extract. *L. crispatus* c2 (♦), *L. salivarus* c4 (●), *L. crispatus* c8 (▲), *L. johnsonii* g4 (■), *L. crispatus* int8 (Δ), *L. reuteri* caeca4 (○), *L. salivarus* caeca6(□), *L. crispatus* caeca10 (◊)

in n-hexen (Table 4). Adherence to the epithelial cell was positive in five out of eight species, based on the defined criteria (Table 4).

Hydrogen peroxide production test

The ability of H_2O_2 production was observed in all strains except for *L. reuteri* caeca4. Due to intensity of the blue color of colonies, *L. salivarus* c4*, L. salivarus* caeca6, and *L. crispatus* caeca10 were detected strong H_2O_2 producers.

DISCUSSION

The probiotic potential of lactobacilli was evaluated by several *in vitro* tests. Since the aim of this study was to introduce a probiotic strain for chickens, the lactobacilli were isolated from the chicken gut. Among numerous

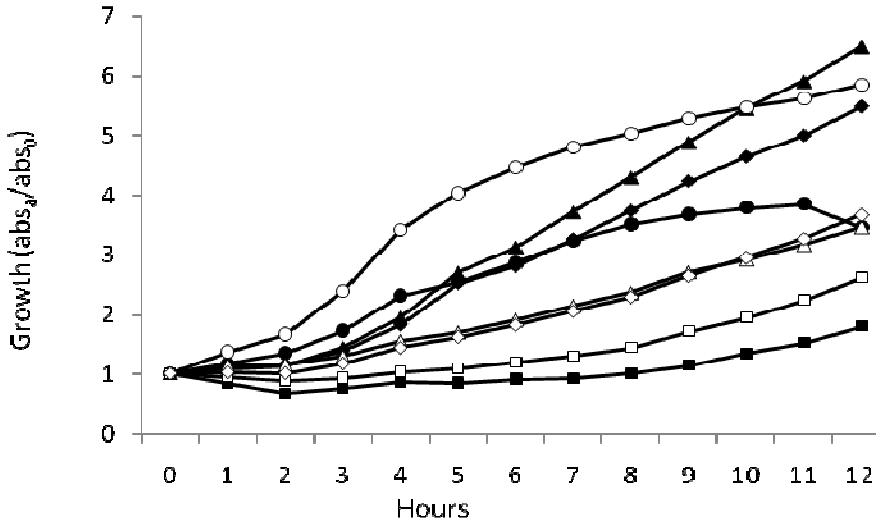

Figure 5. Growth of lactobacilli in MRS broth contain 21 mmol l^{-1} sodium taurocholate. *L. crispatus* c2 (♦), *L. salivarus* c4 (●), *L. crispatus* c8 (▲), *L. johnsonii* g4 (■), *L. crispatus* int8 (Δ), *L. reuteri* caeca4 (○), *L. salivarus* caeca6 (□), *L. crispatus* caeca10 (◊).

Table 4. Hydrophobicity and adhestion test for selected lactobacilli.

Strain	Hydrophobicity (%)†		Adhesion ‡	H_2O_2 production
	n-hexadecan	xylene		
L. crispatus c2	76.9	89.36	< 10	+
L. salivarus c4	91.9	86.64	> 10	+
L. crispatus c8	90.5	93.08	< 10	+
L. johnsonii g4	94.53	91.64	> 10	+
L. crispatus int8	75.22	92.54	> 10	+
L. reuteri caeca4	89.64	93.20	> 10	-
L. salivarus caeca6	87.37	78.21	< 10	+
L. crispatus caeca10	89.5	87.93	> 10	+

† Percent of adhesion to non polar solvents, ‡ number of adhered lactobacilli to a crop epithelium cell.

existing tests, the auto-aggregation test has become known as a criterion for the selection of bacteria (Ehrmann et al., 2002) since this factor shows the ability of lactobacilli to interact with the pathogenic bacteria (Gusils et al., 1999). In this study, lactobacilli with short auto-aggregation time were selected as this criterion is necessary for a convenient competitive exclusion effect.

Salmonella and *E. coli* are common pathogenic bacteria that threaten the safety of GI tract in human and animal. It is reported that lactobacilli have inhibitory activity against pathogen bacteria including *salmonella* and *E. coli* (Miyamoto et al., 2000; Garriga et al., 1898). *L. salivarus* c4 and *L. crispatus* caeca10 had the highest inhibitory activity in this study. This result correspond to other studies that observed a significant decrease in the viability of *Salmonella* or *E. coli* in present of this two species (Murry et al., 2004a; Nouri et al., 2010). Environment of GI tract is suitable for growing of pathogenic bacteria if pH of GI tract goes toward the basic (Payne et al., 2007; Presser et al., 1997). In this study, decreasing of pH inhibited the growth of *E. coli*. Inhibitory zones were seen more in strains that pH of their supernatant reach to 4 or less ($p<0.05$) (Table 2). There was a significant linear correlation between supernatant pH and inhibition of *E. coli* ($R= -0.55$)($p<0.05$). The results concurrent with previous studies that showed lactobacilli can protect GI from pathogenic bacteria by decreasing the environmental pH (Murry et al., 2004a; Taheri et al., 2009). No significant correlation between pH of supernatant and *Salmonella* inhibition was observed ($R= -0.19$).

The tolerance to acidity seems to be an important trait of probiotic strains if there is no facility for their

Table 5. Total score of lactobacilli were obtained from different tests.

Strain	*L.crispatus* c2	*L.salivarus* c4	*L.crispatus* c8	*L.johnsonii* g4	*L.crispatus* int8	*L.reuteri* cecu4	*L.salivarus* cecu6	*L.crispatus* cecu10
Total score	11.207	22.479	12.909	9.34	10.809	10.922	8.129	17.215

encapsulation. Generally, growth and fermentation of lactobacilli create an acidic condition and they are resistant to acidic conditions. As presented in Table 4, *L. johnsonii* g4 and *L. salivarius* c4 exhibit a low tolerance to an acidic environment, as mentioned in various surveys (Sheehan et al., 2007; Belkacem et al., 2009). It is suggested that *L. salivarius* is suitably acid resistant in gastric juice (Kato et al., 2008), however it is assumed that some component in gastric juice may confer some protective effects on the bacterial cells (Conway et al., 1987). Therefore, during acid resistance evaluation test, it was reasonable to use the viability data in PBS as an index. In this study *L. crispatus* c8 and *L. reuteri* caeca4 showed more tolerance in critical pH than other species. These results confirmed by others who indicated that these species are acceptably viable in acidic condition (Jin et al., 1998; Ehrmann et al., 2002; Taheri et al., 2009; Belkacem et al., 2009; Chang et al., 2001; So-Young et al., 2006).

The secretion of bile extract into the duodenum directly hampers probiotic bacteria. Bile acids are amphipathic molecules with antimicrobial potential that act as a detergent and interfere with biological membranes (Lebeer et al., 2008). The toxicity effects of bile acids on bacterial cells has not been well known. In this study, *L. crispatus* c8 and *L. salivarus* c4 were the most resistant strains to bile salts (Figure 3). In contrast with other species, *L. crispatus* c8 grew and proliferated in a medium contains 0.3% ox bile. The resistance of *L. crispatus* to bile salt has been reported, previously (Jin et al., 1998; Moser and Savage, 2001). This ability is originated from the production of bile salt hydrolase enzyme. Moser and Savage (2001) confirmed the existence of the enzyme in *L. crispatus*. The bile salt hydrolase enzyme is able to deconjugate bile salt to amino acids and cholesterol which lead to the reduction of the toxicity of bile acids on bacteria (De Smet et al., 1995).

The response of bacteria to taurocholate presence in this study was found variable (Figure 5). Previous studies have shown that there are two kinds of bile salt hydrolase enzymes in lactobacilli, including taurodeoxycholic acid hydrolase and taurocholic acid hydrolase. The ability of these two enzymes in hydrolyzing bile salts is different (Moser and Savage, 2001). Therefore, the differences in the response of bacteria toward taurocholate were observed in this study may reflect from the variety of enzyme function.

Hydrophobicity and adhesion properties define the adherence of bacterium to epithelial cells. Since *Salmonella* and other intestinal pathogens must adhere to the epithelium for invading and multiplying, property of adhesion of probiotic is very important (Martin-Pelaez and Martin-Orue, 2009). There are many studies which have confirmed a positive correlation between hydrophobicity and adhesion (Li et al., 2008; Rosenberg et al., 1983; Ehrmann et al., 2002) and between aggregation and hydrophobicity (Bujnakova et al., 2004; Rahman et al., 2008). Therefore, it is reasonable to assume that bacteria with an optimum auto-aggregation property have higher hydrophobicity and adhesion. In this study, the selection of bacterial strain with short auto-aggregation time strongly showed high percent hydrophobicity and adhesion (Table 4). *L. crispatus* c8 and *L. reuteri* caeca4 had the most hydrophobicity that this result is match to Taheri et al. (2009) and Ehrmann et al. (2002).

Decision for selection of ideal strain for probiotic purposes might be difficult regard to multiple criteria. Characterization of criteria are useful based on their importance for selection of the best bacteria as a probiotic. In this study, it is defined a coefficient for each criterion, named “Importance Coefficient” that was multiplied to the result of the test for each strain. The summation of multiples makes final score for each strain. The criteria that involve for competitive exclusion or inhibitory have high coefficient (coefficient=2 and 3 respectively) and bile salt resistance (coefficient=2). Since the pH sensitivity could be eliminated by encapsulation of bacteria, low coefficient was given to it (coefficient=1.5). Base on these definitions, all isolated lactobacillus were scored (Table 5). *L. salivarius* c4 (score =22.4) showed the highest score among other strains. *L. crispatus* caeca10 (score= 17.2) got second place in score order. *L. salivarius* c4 showed an acceptable inhibitory activity against *Salmonella* and *E. coli* and suitable resistance against bile salt. *L. crispatus* caeca10 exhibited satisfactory hydrophobicity and antibacterial property against *E. coli*. In this study, from native lactobacilli of the GI tract, *L. salivarius* c4 demonstrated a suitable resistance to bile salt inhibitory activity against *Salmonella* and *E. coli*.

These abilities along with the higher total scores make *L. salivarius* c4 and *L. crispatus* caeca10 for probiotic purposes. Further experiments are necessary to evaluate the *in vivo* properties of these strains.

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REFERENCES

Belkacem B, Meriem M, Mebrouk K (2009). Probiotic Potential of Thermotolerants Lactobacilli Isolated from Chicken Gastrointestinal Digestive and Their Use as Poultry Feed. World Appl. Sci., 7: 951-957.

Bujnakova D, Vlkova E, Rada V, Kmet V (2004). Aggregation of Lactobacilli and Bifidobacteria with *Escherichia coli* O157. Folia Microbiol (Praha), 49: 143-146.

Busarcevic M, Kojic M, Dalgalarrondo M, Chobert JM, Haertle T, Topisirovic L (2008). Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1. Oral. Microbiol. Immunol., 23: 254-258.

Chang YH, Kim JK, Kim HJ, Kim WY, Kim YB, Park YH (2001). Selection of a potential probiotic *Lactobacillus* strain and subsequent in vivo studies. Antonie Van Leeuwenhoek, 80: 193-199.

Conway PL, Gorbach SL,Goldin BR (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci., 70: 1-12.

De Smet I, Van Hoorde L, Vande Woestyne M, Christiaens H, Verstraete W (1995). Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol., 79: 292-301.

Dunne C, Murphy L, Flynn S, O'mahony L, O'halloran S, Feeney M, Morrissey D, Thornton G, Fitzgerald G, Daly C, Kiely B, Quigley EM, O'sullivan GC, Shanahan F, Collins JK (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek, 76: 279-292.

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res., 17: 7843-7853.

Ehrmann MA, Kurzak P, Bauer J, Vogel RF (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J. Appl. Microbiol., 92: 966-975.

Eschenbach DA, Davick PR, Williams BL, Kleibanoff SJ, Young-Smith K, Critchlow CM, Holmes KK (1989). Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J. Clin. Microbiol., 27: 251 - 256.

Fuller R (1973). Ecological studies on the *Lactobacillus flora* associated with the crop epithelium of the fowl. J. Appl. Bacteriol., 36: 131 - 139.

Garriga M, Pascual M, Monfort JM, Hugas M (1898). Selection of lactobacilli for chicken probiotic adjuncts. J. Appl. Microbiol., 84: 125-132.

Gusils C, Perez Chaia A, Gonzalez S, Oliver G (1999). Lactobacilli isolated from chicken intestines: potential use as probiotics. J. Food Prot., 62: 252-256.

Hilmi HTA, Surakka A, Apajalahti J, Saris EJ (2007). Identification of the Most Abundant *Lactobacillus* Species in the Crop of 1- and 5-Week-Old Broiler Chickens. Appl. Environ. Microbiol., 73: 7867–7873.

Jin LZ, Ho YW, Abdullah N, Jalaludin S (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. Lett. Appl. Microbiol., 27: 183-185.

Kalavathy R, Abdullah N, Jalaludin S, Wong CMVL, Ho YW (2008). Effect of *Lactobacillus* Cultures and Oxytetracycline on the Growth Performance and Serum Lipids of Chickens. Int. J. Poult. Sci., 7: 385 - 389.

Kalavathy R, Abdullah N, Jalaludin S, Wong MCVL, Ho YW (2006). Effects of *Lactobacillus* feed supplementation on cholesterol, fat content and fatty acid composition of the liver, muscle and carcass of broiler chickens. Anim. Res., 55: 77 - 82.

Kato A, Nakaya S, Suzuki N (2008). Probiotic products containing *L. salivarius* strain. Wakamoto Pharmaceutical Co., Ltd. (5-3, Nihonbashi-Muromachi 1-chome, Chuo-ku, Tokyo, pp. 103-8330.

Kizerwetter-Swida M, Binek M (2009). Protective effect of potentially probiotic *Lactobacillus* strain on infection with pathogenic bacteria in chickens. Pol. J. Vet. Sci., 12: 15-20.

Lan PTN, Sakamoto M, Benno Y (2004). Effects of Two Probiotic *Lactobacillus* Strains on Jejunal and Cecal Microbiota of Broiler Chicken under Acute Heat Stress Condition as Revealed by Molecular Analysis of 16S rRNA Genes. Microbiol. Immunol., 48: 917-929.

Lebeer S, Vanderleyden J, De Keersmaecker SC (2008). Genes and molecules of lactobacilli supporting probiotic action. Microbiol. Mol. Biol. Rev., 72: 728-764.

Li XJ, Yue LY, Guan XF, Qiao SY (2008). The adhesion of putative probiotic lactobacilli to cultured epithelial cells and porcine intestinal mucus. J. Appl. Microbiol., 104: 1082-1091.

Martin-Pelaez S, Martin-Orue S (2009). Feeding strategies for the control of *Salmonella* in pigs In: Gibson Glenn R (eds) Food Science and Technology Bulltein: Functional food. IFIS publishing, Shinfield, pp. 39-50.

Mead GC (1997). Bacteria in the gastrointestinal tract of birds. In: Mackie RJ, White BA & Isaacson RE (eds) Gastrointestinal Microbiology. Chapman and Hall, New York, pp. 216-240.

Miyamoto T, Horie T, Fujiwara T, Fukata T, Sasai K, Baba E (2000). *Lactobacillus* Flora in the Cloaca and Vagina of Hens and Its Inhibitory Activity Against *Salmonella enteritidis In vitro*. Poult. Sci., 79: 7-11.

Moser SA, Savage DC (2001). Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. Appl. Environ. Microbiol., 67: 3476-3480.

Murry AC, Hinton A, Morrison H (2004a). Inhibition of Growth of *Escherichia coli*, *Salmonella typhimurium*, and *Clostridia perfringens* on Chicken Feed Media by *Lactobacillus salivarius* and *Lactobacillus plantarum*. Int. J. Poult. Sci., 3: 603-607.

Murry AC, Hinton JA, Buhr RJ (2004b). Effect of a Probiotic Containing Two *Lactobacillus* Strains on Growth Performance and Population of Bacteria in the Ceca and Carcass Rinse of Broiler Chickens. Poult. Sci., 83: 322.

Nouri M, Rahbarizadeh F, Ahmadvand D, Moosakhani F, Sadeqzadeh E, Lavasani S, Vishteh VK (2010). Inhibitory effects of *Lactobacillus salivarius* and *Lactobacillus crispatus* isolated from chicken gastrointestinal tract on *Salmonella enteritidis* and *Escherichia coli* growth. Iranian J. Biothech., 8: 32-37.

Pandey A, Franco SG, Soccol CR, Reque EDF (2000). Isolation, Identification and physiological study of *Lactobacillus fermentum* LPB for use as probiotic in chickens. Braz. J. Microbiol., 31: 303-307.

Pascual M, Hugas M, Badiola JI, Monfort JM, Garriga M (1999). *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol., 65: 4981-4986.

Payne JB, Osborne JA, Jenkins PK, Sheldon BW (2007). Modeling the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and water activity. Poult. Sci., 86: 191-201.

Pelletier CBC, Cayuela C, Bouttier S, Bourlioux P, Bellon-Fontaine Mn (1997). Cell surface characteristics of *Lactobacillus casei* subsp. casei, *Lactobacillus paracasei* subsp. paracasei, and *Lactobacillus* rhamnosus strains. Appl. Environ. Microbiol., 63: 1725 - 1731.

Presser KA, Ratkowsky DA, Ross T (1997). Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Appl. Environ. Microbiol., 63: 2355-2360.

Rahman M, Kim WS, Kumura H, Shimazaki K (2008). Autoaggregation

and surface hydrophobicity of bifidobacteria. World J. Microbiol. Biotechnol., 24: 1593–1598.
Reniero R, Cocconcelli P, Bottazzi V, Morelli L (1992). High frequency of conjugation in *Lactobacillus* mediated by an aggregation promoting factor. J. Gen. Microbiol., 138: 763 - 768.
Rosenberg M, Judes H, Weiss E (1983). Cell surface hydrophobicity of dental plaque microorganisms *in situ*. Infect Immun., 42: 831-834.
Russell SM, Grimes JL (2009). The effect of a direct-fed microbial (Primalac) on turkey live performance. J. Appl. Poult. Res., 18: 185–192.
SAS Institute (2004). SAS/STAT User's Guide: Statistics. Version 9.2 Edition. SAS Inst. Inc., Cary, NC.,
Schillinger U, Lucke FK (1989). Antibacterial activity of Lactobacillus sake isolated from meat. Appl. Environ. Microbiol., 55: 1901-1906.
Sheehan VM, Sleator RD, Hill C, Fitzgerald GF (2007). Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bifidobacterium breve* UCC2003. Microbiology, 153: 3563-3571.
So-Young K, Yasuki O, Hiromi N, Yoshikazu A (2006). Heat Resistance, Acid and Bile Tolerance of Canine Intestinal Lactic Acid Bacteria and Bifidobacteria. Japanese J. Anim. Hyg., 32: 5-11.
Taheri HR, Moravej H, Tabandeh F, Zaghari M, Shivazad M (2009). Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poult. Sci., 88: 1586-1593.
Tannock GW (1999). Identification of lactobacilli and bifidobacteria. Mol. Biol., 1: 53-64.