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# Isolation and Characterization of *Lactobacillus* Strains as Potential Probiotics for Chickens

**Shokryazdan, P.[1], Kalavathy, R.[2], Sieo, C. C.[1,3], Alitheen, N. B.[3], Liang, J. B.[4], Jahromi, M. F.[4] and Ho, Y. W.[1]***

[1]*Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Faculty of Pharmacy, Universiti Teknologi MARA, 40450 Shah Alam,Selangor, Malaysia*
[3]*Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

## ABSTRACT

The ban on the use of antibiotics as growth promoters for poultry production in many countries has led to increasing interest to use probiotics as an alternative. In the present study, some *Lactobacillus* strains were isolated from chicken intestines, identified and assessed (*in vitro*) for their ability to survive and colonize the gastrointestinal tract (GIT), with a view to select suitable strains as potential probiotic candidates for chickens. Out of 42 isolated strains, three isolates, identified as *Lactobacillus* strains based on initial identification and tolerant to acid and bile based on preliminary screening using turbidity (optical density) as a measurement of growth, were selected for detailed identification and further *in vitro* assays. The three isolates were identified to species level using carbohydrate fermentation profile analysis and 16S rRNA gene sequencing. Results showed that all three strains belonged to *Lactobacillus salivarius*. The three *L. salivarius* strains were then assessed for their ability to tolerate the stress conditions in the GIT and capacity to adhere to the intestinal epithelial cells using *in vitro* assays of acid, bile and pancreatic enzyme tolerance measured by viable colony counts, and adhesion assay using Caco-2 cell line. The results showed that all three *L. salivarius* strains exhibited good tolerance to acid, bile and pancreatic enzymes and a strong ability to adhere to intestinal epithelial cells. Thus, they would be able to survive the stress conditions of GIT, as well as to attach and colonize the GIT, and could be considered as good potential candidates for probiotics of chickens.

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*E-mail addresses*:
parisa_shokryazdan@yahoo.com (Shokryazdan, P.),
kalav922@puncakalam.uitm.edu.my (Kalavathy, R.),
cssieo@upm.edu.my (Sieo, C. C.),
noorjahan@upm.edu.my (Alitheen, N. B.),
jbliang@upm.edu.my (Liang, J. B.),
mfjahromi@yahoo.com (Jahromi, M. F.),
ywho@upm.edu.my (Ho, Y. W.)
* Corresponding author

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## INTRODUCTION

The poultry industry is one of the most important livestock industries in many countries, including Malaysia, and it contributes substantially to the economies of the countries. Prevention and control of poultry diseases would be necessary in order to avoid large economic losses. For over fifty years, antibiotics were routinely used to prevent or control diseases and to promote growth and feed efficiency (Kabir, 2009). However, with increasing concerns on the emergence of antibiotic resistant bacteria, the rampant use of antibiotics as a preventive tool for diseases and growth promotion was questioned (Patterson & Burkholder, 2003). The ban on subtherapeutic antibiotic usage for livestock production in Europe and the potential ban in the United States have led to an increasing interest in finding alternatives for antibiotics as growth promoters. Probiotics have been considered as one of the alternatives (Kabir, 2009). A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). Every bacterial strain must have some special properties to be considered as a potential probiotic (FAO/WHO, 2001).

Lactobacilli are a group of bacteria that are frequently used as probiotics. They have a long history in their use as probiotics in the food industry and the *Lactobacillus* strains are "generally recognized as safe" (GRAS). They are commonly found in the environment such as soil, water, decaying plant materials, as well as in the normal microflora of the gastrointestinal tract (GIT) of animals (Kizerwetter-Swida & Binek, 2005). They have been used extensively in the food industry as starters in fermented products. In chickens, *Lactobacillus* strains are able to establish in the GIT within a day after hatching as they have a high ability to attach to the intestinal epithelial cells (Fuller, 1973). It has been suggested that in order to have more specific applications, bacterial strains intended as probiotics for chickens should be isolated from the natural microflora of the GIT of chickens (Kizerwetter-Swida & Binek, 2005).

As a result of the growing interest in probiotics, many purported probiotic products have been marketed without proper studies on the probiotic properties of the strains, giving rise to problems of inconsistent efficacy of the products. Several studies have reported misidentification or mislabelling of probiotic species or presence of unspecified species in many commercial probiotic products (Hamilton-Miller & Shah, 1996; Canganella *et al.,* 1997; Klein *et al.,* 1998; Hamilton-Miller *et al.,* 1999; Schillinger, 1999). In Malaysia, one of the reasons for the reluctance in the use of probiotics as an alternative to antibiotic growth promoters by poultry farmers is the inconsistency of the probiotic products' efficacy. Since the properties of probiotic are strain specific, the quality of products is closely linked to the individual strains in the products, thus, they should

be correctly identified and their probiotic properties properly studied. In 2001, FAO/WHO produced a set of guidelines for the evaluation of probiotics in food in which they recommended that every potential probiotic strain must be correctly identified, followed by various *in vitro* assays to investigate its functional properties and *in vivo* trials for its safety. This is because probiotic properties are strain specific and cannot be extrapolated to the whole genus or species. In the present study, some *Lactobacillus* strains were isolated from chicken intestines, identified and characterized for their probiotic properties, with a view to select suitable strains with probiotic attributes as potential probiotic candidates for chickens. The *Lactobacillus* strains were identified to species level using phenotypic and molecular characteristics and the primary probiotic properties (recommended by FAO/WHO, 2001) studied (*in vitro*) were the ones which would enable the strains to survive and colonize the GIT such as the abilities to tolerate acid, bile and pancreatic enzymes, and the capacity to adhere to the intestinal epithelial cells.

## MATERIALS AND METHODS

### *Isolation of the Lactobacillus Strains*

Samples of intestinal contents were collected from five healthy chickens at 42 days of age from the farm of the Department of Animal Science, Universiti Putra Malaysia. Chicken intestines were collected aseptically immediately after the chickens were euthanized. Intestinal tissues were washed with sterile phosphate buffer saline (PBS) (8g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$ in 1 l distilled water, pH 7.2) to remove intestinal contents and surface mucus to obtain adhering bacteria. Intestinal epithelial tissues were scraped with a sterile blade to obtain 1 g of content. Then, 10-fold serial dilutions of up to $10^{-8}$ for each sample were prepared using sterile diluent (0.5% peptone in distilled water). From each dilution of $10^{-2}$ to $10^{-8}$, 100 µl was streaked onto sterile petri dishes containing de Man, Rogosa and Sharpe (MRS) agar medium (Merck) and incubated for 72h at 37°C in anaerobic jars (Oxoid) containing gaspack (AnaeroGen, Oxoid). After incubation, well-formed colonies were randomly picked and streaked onto new MRS plates. The isolates were purified by subculturing them three times on MRS agar. Meanwhile, the stock cultures of pure isolates were stored in 20% glycerol at -80°C, and the cultures for studies were maintained routinely on MRS agar.

### *Initial Identification and Preliminary Screening of Isolates*

For initial identification of the selected isolates, morphological examination, Gram staining and catalase test were performed. Overnight cultures of each isolate on MRS agar were used. The isolates were Gram stained and examined under a light microscope (Dialux, Leitz Wetzlar, Germany) for morphological characteristics. In the catalase test, 50 µl of 3% hydrogen peroxide was dropped on randomly selected colonies of each isolate on MRS agar. Effervescence from the colonies indicated

positive reaction. Only gram-positive and catalase-negative isolates (Kandler & Weiss, 1986; Schillinger & Lucke, 1987) were selected for a rapid preliminary screening of their acid and bile tolerance [growth measured as increase in turbidity determined by optical density (OD)] to select only those with good tolerance levels for detailed identification and further *in vitro* assays of their probiotic characteristics for survival in the stress conditions of GIT.

The preliminary screening for acid tolerance was according to Ehrmann *et al*. (2002) with modifications. Bacterial cells from overnight cultures in MRS broth (Merck) (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS and resuspended in sterile PBS at a final concentration of 7 to 8 log CFU/ml. The resuspended cells were then inoculated (1%, v/v) into PBS adjusted to pH 1, 2 and 3 with 1 N HCl (acidic condition) and normal PBS with pH 7.2 (control). After 3 h of incubation at 37˚C, 1% (v/v) of cell suspension was inoculated into 10 ml of fresh MRS broth and incubated at 37˚C for 24 h. After the incubation period, cell growth was assessed by measuring OD at 620 nm using a spectrophotometer (Barnstead International, USA). The isolates that showed at least 80% growth, in comparison with that of the control (100%), were selected as good acid tolerant strains. Three replicates were made for each isolate at each pH value. The experiment was carried out twice.

For the preliminary screening of bile tolerance, only the isolates that showed good acid tolerance were used. The bile tolerance test followed that of Jacobsen *et al.* (1999) with modifications. The overnight culture of each isolate (adjusted to a final concentration of 7 to 8 log CFU/ml) was inoculated (1%, v/v) into 10 ml of fresh MRS broth with or without (control) 0.3% oxgall (Sigma) and incubated at 37˚C for 4 h. After incubation, growth was assessed by measuring OD at 620 nm. The isolates that showed at least 80% growth, in comparison with that of the control (100%), were selected as good bile tolerant strains. Three replicates were made for each isolate and the experiment was carried out twice.

*Identification of the Isolated Strains to Species Level*

The isolates selected for their good acid and bile tolerance were identified to species level using a biochemical method [carbohydrate fermentation profile analysis by API system (Bio-Merieux)] and a molecular technique (comparative sequence analysis of the 16S rRNA gene). For identification using the API system, the overnight culture of each isolate on MRS agar was used and carbohydrate fermentation profiles of the isolates were investigated using API 50 CH kits according to the manufacturer's instructions. The strains were identified using API LAB Plus software version 3.3.2 (Bio-Merieux).

For 16S rRNA gene sequencing, the cells of overnight cultures of each isolate in MRS broth were harvested by centrifugation at 5000 x *g* for 10 min at room temperature and used for DNA extraction. The DNeasy Blood & Tissue Kit

(QIAGEN, Germany) was used to extract total DNA according to the manufacture's instructions. For amplification of the 16S rRNA gene, two universal primers, F27 (AGAGTTTGATCMTGGCTCAG) and R1492 (TACGGYTACCTTGTTACGACTT) were used (Lane, 1991; McDonald *et al.,* 1995), with the expected PCR product of 1.5 kb. The PCR amplification was performed in 50 µl reaction mixtures using a MyCycler Thermal Cycler (Bio-Rad, USA), as described by McDonald *et al.* (1995). The PCR conditions were initial denaturation at 94˚C for 4 min, 30 cycles of denaturation at 94˚C for 1 min each, annealing at 55˚C for 30 s, and at 72˚C for 2 min, and a final extension at 72˚C for 5 min. The PCR-amplified product was electrophorized (80 v, 50 min) on a 1% (w/v) horizontal agarose gel, followed by staining with ethidium bromide (2 µg/ml) for 10 min, and visualizing using an Alpha Imager Documentation and Analysis System (Alpha Innotech, USA). The PCR product with the expected size of 1.5 kb was excised and purified using MEGAquick-spin$^{(TM)}$ PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Korea). Then, each purified PCR product was cloned into *E.coli* plasmid using a TOPO TA cloning kit (Invitrogen, USA). Colonies containing the 16S rRNA gene inserts were screened, picked and cultured in Luria-Bertani broth. Plasmid extraction was performed using a DNA-Spin Plasmid DNA Extraction kit (iNtRON Biotechnology, Korea). DNA sequence analysis was carried out for plasmid with the unique insert using an ABI 373XL automated sequencer (Applied Biosystems, USA) at both directions to obtain the full sequence of the amplicons.

*Sequence Alignments and Phylogenetic Inference*

DNA sequence data sets were assembled using the Bioedit sequence alignment editor software, version 7.0.9.0 (Hall, 1999). Discrepancy nucleotides between forward and reverse sequences were edited based on their electropherograms. Similarity values were determined using the Basic Local Alignment Search Tool (BLAST) of the GenBank (NCBI). Sequences with ≥ 97% similarity to the previously published sequences were used as the criterion to indicate species identity (Stackebrandt & Goebel, 1994).

A phylogenetic tree was constructed based on the 16S rRNA gene sequence analysis, in which the analysis involved 25 nucleotide sequences consisting of 3 sequences of *Lactobacillus* strains obtained in this study, 21 sequences belonging to *Lactobacillus* species obtained from the GenBank and the sequence of *Lactococcus lactis* (AB 100803.1) which was used as the outgroup. Evolutionary analyses were conducted with MEGA5 software (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). Bootstrapping was performed for 1000 replicates and only bootstrap values (the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test) above 50%

were reported (Felsenstein, 1985). The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992). Only values above 70 to 80% are usually considered to indicate high support; in this study, the values above 50% were considered to avoid dispersion of species. Potential anormalous sequences of the 16S rRNA gene were examined by the Mallard (Ashelford *et al.,* 2005) and the Bellerophon (Huber *et al.,* 2004) programs. Nucleotide sequences determined in this study were deposited in the public database (GenBank, NCBI) using the Sequin program.

*Characterization of Selected Lactobacillus Strains for Survival in the Gastrointestinal Tract*

*In vitro* assays for acid, bile and pancreatic enzyme tolerance were employed to characterize the selected *Lactobacillus* strains for survival in the GIT. Although acid and bile tolerance of the strains had been carried out in the preliminary screening, growth was estimated as increase in turbidity, which was good for rapid screening of a large number of strains, but was not very accurate as it measured dead cells along with live cells. As acid and bile tolerance is a prerequisite for survival in the GIT, in the present *in vitro* assays, growth was measured as viable cells [colony forming units (CFU)] counts (CFU/ml), which is a more accurate measurement of growth. A probiotic *Lactobacillus* strain, *L. reuteri* C10, from a commercial multi-strain probiotic (StellarLac) for chickens, was used as a reference strain. This strain was kindly provided by Stellar Gen Ltd Co. The growth medium for all *Lactobacillus* strains was MRS agar or broth, and the cultures were incubated anaerobically at 37˚C.

***In vitro* assay for acid tolerance**

The acid tolerance assay followed that of Ehrmann *et al.*, (2002) with modifications. Bacterial cells from overnight cultures (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), then resuspended in sterile PBS adjusted to a final concentration of 7 to 8 log CFU/ml. The resuspended cells were inoculated (1%, v/v) into sterile PBS adjusted to pH 3 with 1 N HCl (acidic condition) and normal PBS with pH 7.2 (control), and incubated anaerobically for 3 h at 37˚C. After incubation, 10-fold serial dilutions (up to $10^{-7}$) of each *Lactobacillus* strain were prepared using PBS. Then 100 µl of $10^{-4}$ to $10^{-7}$ dilutions from each sample was streaked on MRS agar plates and incubated anaerobically at 37˚C for 24 h. After incubation, viability of bacterial cells was assessed by colony counts (CFU/ml) on the plates (Jacobsen *et al.* 1999; Ehrmann *et al.* 2002; Paramithiotis *et al.* 2006; Bilige *et al.* 2009). Tolerance to acidic condition was estimated by comparing viable cell counts after exposure to acidic (pH 3) and normal (control) conditions. The assay was performed twice, each in triplicate.

***In vitro* assay for bile tolerance**

The bile tolerance assay was according to Jacobsen *et al.* (1999) with modifications. Overnight culture of each *Lactobacillus*

strain (adjusted to a final concentration of 7 to 8 log CFU/ml) was inoculated (1%, v/v) into 10 ml of fresh MRS broth with or without (control) 0.3% (w/v) oxgall (Sigma, USA) and incubated anaerobically at 37˚C for 4 h, after which 10-fold serial dilutions of up to $10^{-7}$ were prepared using PBS. Then 100 µl of $10^{-4}$ to $10^{-7}$ dilutions from each sample was streaked on MRS agar plates and incubated anaerobically at 37˚C for 24 h. After incubation, colonies on the plates were counted and enumerated as CFU/ml (Gilliland *et al.* 1984; Jacobsen *et al.* 1999; Paramithiotis *et al.* 2006). Bile tolerance was estimated by comparing viable cell counts in MRS with and without bile (oxgall). The assay was performed twice, each in triplicate.

### *In vitro* assay for pancreatic enzyme tolerance

Tolerance to pancreatic enzymes was tested according to the method of Ronka *et al.* (2003) with modifications. Bacterial cells from overnight cultures (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), and resuspended in sterile PBS at a final concentration of 7 to 8 log CFU/ml. The resuspended cells were inoculated (1%, v/v) into 10 ml of the test solution [PBS containing 150 mM $NaHCO_3$ and 1.9 mg/ml pancreatin (Sigma); pH 8] and control solution (PBS, pH 7.2). The cultures were incubated anaerobically at 37˚C for 3 h. After incubation, 10-fold serial dilutions of up to $10^{-7}$ were prepared using PBS, and 100 µl of $10^{-4}$ to $10^{-7}$ dilutions from each sample was streaked on MRS agar plates. The plates were incubated anaerobically at 37˚C for 24 h, after which viability of bacterial cells was estimated by colony counts (CFU/ml). Tolerance to pancreatic enzymes was estimated by comparing viable cell counts of test solution and control solution. The assay was performed twice, each in triplicate.

### *Adhesion assay*

The human intestinal epithelial cell line, Caco-2 cell line (ATCC 2102-CRL), purchased from the American Type Culture Collection (ATCC), was used in the adhesion assay. The Caco-2 cells were routinely grown to 80 to 85% confluent in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 20% (v/v) fetal bovine serum (FBS) (Sigma), 10000 IU/ml penicillin (Sigma) and 10 mg/ml streptomycin (Sigma). The procedure used for the adhesion assay followed that of Gopal *et al.* (2001) with modifications. A cell suspension ($1\times10^5$ cell/ml DMEM) of Caco-2 cells was used for preparation of a monolayer of the cells on glass cover slips placed in six-well tissue culture plates. One ml of the cell suspension was added into each well of the plates containing fresh DMEM, and the plates were incubated overnight. Incubation for maintenance of cells and adhesion assay was at 37˚C in 5% $CO_2$. For each *Lactobacillus* strain, cells from overnight culture (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), then resuspended

in sterile PBS buffer (pH 7.2) to a final concentration of $1\times10^8$ CFU/ml. Adherence assay was performed by adding 100 µl of bacterial suspension onto the washed (once with PBS) monolayer of Caco-2 cells in the well containing 2 ml of fresh DMEM and incubated for 1 h at 37˚C. After incubation, the monolayers were washed four times with PBS to remove unattached bacteria, fixed with 3 ml of methanol and incubated for 5 to 10 min at room temperature. The fixed monolayers were Gram stained and examined with a light microscope under oil immersion lens (Dialux, Leitz Wetzlar). Adherence was evaluated in 20 random microscopic fields and the number of adhered *Lactobacillus* cells per Caco-2 cell was determined (Jacobsen *et al.* 1999; Gopal *et al.* 2001; Ali *et al.* 2008; Pan *et al.* 2009). The assay was performed twice, each in triplicate.

*Statistical analysis*

Data of the *in vitro* assays for acid, bile and pancreatic enzyme tolerance were analyzed by one-way analysis of variance using the SAS (Statistical Analysis System, 2008) program version 9.2. Treatment means were compared using Duncan's new multiple range test, and differences were considered significant at $P < 0.05$.

## RESULTS AND DISCUSSION

*Isolation, initial identification and preliminary screening*

A total of 42 bacterial isolates displaying general morphological characteristics of *Lactobacillus* were isolated from chicken intestines. The cell morphologies of the 42 isolates, observed using light microscopy, showed that all 42 isolates were rod shaped, varying from short to long (1.1 to 5.7 µm long), and straight to crescent rods, arranged singly, in pairs or in short or long chains. Catalase test showed that of the 42 isolates, 37 isolates were catalase negative, and of these, 26 isolates were Gram positive. In the preliminary screening of acid tolerance, none of the 26 isolates survived at pH 1 and 2. Sahadeva *et al.* (2011) had also found that none of the tested probiotic strains in their study, which included *L. acidophilus*, *L. casei*, *L. casei* Shirota, *Streptococcus thermophilus* and *Bifidobacterium*, could survive for 3 h at pH 1.5. Earlier, Chan *et al.* (2005) demonstrated that even aciduric *Lactobacillus* strains such as *L. acidophilus* could not survive after 2 h of exposure to pH 2. The results of the preliminary screening showed that at pH 3, 14 of the 26 isolates showed 95.6 to 107.0% growth in comparison with that of the control (100%), which was considered as good acid tolerant isolates, and were then tested for bile tolerance. Of these 14 isolates, only three isolates showed 85.2 to 92.8% growth in the presence of bile when compared to that of the control (100%), and were considered as good bile tolerant isolates. From this preliminary screening, only three isolates, designated as CI1, CI2 and CI3, were selected for detailed identification and further investigation on their ability to survive the stress conditions of the GIT and to adhere to the intestinal epithelial cells.

*Identification using API system and 16S rRNA gene sequencing*

The three isolates were identified based on both phenotypic and genotypic characterizations because according to FAO/WHO (2001) guidelines, a combination of phenotypic and genotypic methods should be used for identification and speciation of probiotic strains. The results of the carbohydrate fermentation profiles of the three isolates are shown in Table 1. All three isolates were able to ferment galactose, glucose, fructose, maltose, mannitol, sorbitol, N-Acetyl-Glucosamine, lactose, melibiose, sucrose and raffinose. The results of identification of the isolates using carbohydrate fermentation profile analysis by the API system and the 16S rRNA gene sequencing are presented in Table 2. Based on the results of carbohydrate fermentation profile analysis by the API system, the three isolates were 98.8 to 99.9% similar to *Lactobacillus salivarius*. The results of the 16S rRNA gene sequencing also showed that all the three isolates were 99% similar to *L. salivarius* DQ444477.1 (from the GenBank) with 99% query coverage. Thus, there were no discrepancies in the identification of the three isolates using the carbohydrate fermentation profile analysis by the API system and using 16S rRNA gene sequencing. However, some studies have shown that the API carbohydrate fermentation profile analysis was not adequate for identification to species level for some *Lactobacillus* strains (Yin *et al.,* 2005; Khunajakar *et al.*, 2008). The 16S rRNA gene sequences of the three isolates (*L. salivarius* CI1, CI2 and CI3) were deposited in the GenBank database under the accession numbers JN188391 to JN188393 (Table 2).

TABLE 1
Carbohydrate fermentation pattern of the three isolated strains

| No. | API CH50 Kit substrate | CI1 | CI2 | CI3 |
|---|---|---|---|---|
| 0 | Control | – | – | – |
| 1 | Glycerol | – | – | – |
| 2 | Erythritol | – | – | – |
| 3 | D-Arabinose | – | – | – |
| 4 | L-Arabinose | – | – | – |
| 5 | Ribose | – | – | – |
| 6 | D-Xylose | – | – | – |
| 7 | L-Xylose | – | – | – |
| 8 | Adonitol | – | – | – |
| 9 | B-Methyl-D-Xyloside | – | – | – |
| 10 | Galactose | + | + | + |
| 11 | Glucose | + | + | + |
| 12 | Fructose | + | + | + |
| 13 | Mannose | + | + | + |
| 14 | Sorbose | – | – | – |
| 15 | Rhamnose | – | – | – |
| 16 | Dulcitol | – | – | – |
| 17 | Inositol | – | – | – |
| 18 | Mannitol | + | + | + |
| 19 | Sorbitol | + | + | + |
| 20 | A-Methyl-D-Mannoside | – | – | – |
| 21 | A-Methyl-D-Glucoside | – | – | – |
| 22 | N-Acetyl-Glucosamine | + | + | + |
| 23 | Amygdalin | – | – | – |
| 24 | Arbutin | – | – | – |
| 25 | Esculin | – | – | – |
| 26 | Salicin | – | – | – |
| 27 | Cellobiose | – | – | – |
| 28 | Maltose | – | – | – |

TABLE 1 (*continue*)

| No. | API CH50 Kit substrate | CI1 | CI2 | CI3 |
|---|---|---|---|---|
| 29 | Lactose | + | + | + |
| 30 | Melibiose | + | + | + |
| 31 | Sucrose | + | + | + |
| 32 | Trehalose | + | _ | _ |
| 33 | Inulin | _ | _ | _ |
| 34 | Melezitose | _ | _ | _ |
| 35 | Raffinose | + | + | + |
| 36 | Starch | _ | _ | _ |
| 37 | Glycogen | _ | _ | _ |
| 38 | Xylitol | _ | _ | _ |
| 39 | Gentiobiose | _ | _ | _ |
| 40 | D-Turanose | _ | _ | _ |
| 41 | D-Lyxose | _ | _ | _ |
| 42 | D-Tagatose | _ | _ | _ |
| 43 | D-Fucose | _ | _ | _ |
| 44 | L-Fucose | _ | _ | _ |
| 45 | D-Arabitol | _ | _ | _ |
| 46 | L-Arabitol | _ | _ | _ |
| 47 | Gluconate | _ | _ | _ |
| 48 | 2-Keto-Gluconate | _ | _ | _ |
| 49 | 5-Keto-Gluconate | _ | _ | _ |

+, positive reaction; –, negative reaction.

Fig.1 shows the phylogenetic tree based on the 16S rRNA gene sequence analysis, depicting the phylogenetic relationships among the three *Lactobacillus* strains and 21 *Lactobacillus* type strains obtained from the GenBank. *Lactococcus lactis* (AB100803.1) was used as the outgroup. Strains CI1, CI2 and CI3, isolated in this study, were clustered together and were monophyletic with *L. salivarius* DQ444477.1 with a bootstrap value of 100%.

### *Acid, Bile and Pancreatic Enzyme Tolerance*

In the case of functional probiotic properties, the ability to tolerate the stress conditions of GIT is one of the most important criteria in the selection of a strain as a potential probiotic candidate (Ouwehand *et al.,* 1999). Among the stress conditions, the presence of acid, bile salts and pancreatic enzymes are the most important stresses which an orally taken probiotic would encounter in GIT. Thus, it is essential that a potential probiotic strain is able to tolerate these stressful conditions in order to survive in GIT.

In chickens, mucous glands only exist near the entrance of the crop, so the intraluminal pH of the crop is relatively basic compared to the pH value in the proventriculus and gizzard (Klasing, 1998), which ranges from 2.5 to 4.74 and food ingestion can take up to 1 to 3 h depending on feed size (Musikasang *et al.,* 2009). In many studies, pH 3 has been considered as a standard pH for investigation of acid

TABLE 2
Identification using API system and 16S rRNA gene sequencing

| Isolate | Identification using API 50 CH | | Identification using 16 S rRNA | | |
|---|---|---|---|---|---|
| | Nearest matched species | Similarity (%) | Nearest matched species from GenBank | Similarity (%) | Accession number |
| CI1 | *L. salivarius* | 99.9% | *L. salivarius* (DQ444477.1) | 99% | JN188391 |
| CI2 | *L. salivarius* | 98.8% | *L. salivarius* (DQ444477.1) | 99% | JN188392 |
| CI3 | *L. salivarius* | 98.8% | *L. salivarius* (DQ444477.1) | 99% | JN188393 |

tolerance of probiotic strains (Liong & Shah, 2005; Koll *et al.,* 2008; Sahadeva *et al.,* 2011). In view of these studies and the results of the preliminary screening of the isolated strains in the present acid tolerance assay, in which none of the strains was able to survive at pH 1 and 2, only pH 3 was used to investigate the acid tolerance of the three *L. salivarius* strains.

The results of the acid tolerance assay (Table 3) showed that all three *L. salivarius* strains could tolerate pH 3 for 3 h. However, *L. salivarius* CI1 and CI3, with 0.05 log units reduction in cell viability, exhibited

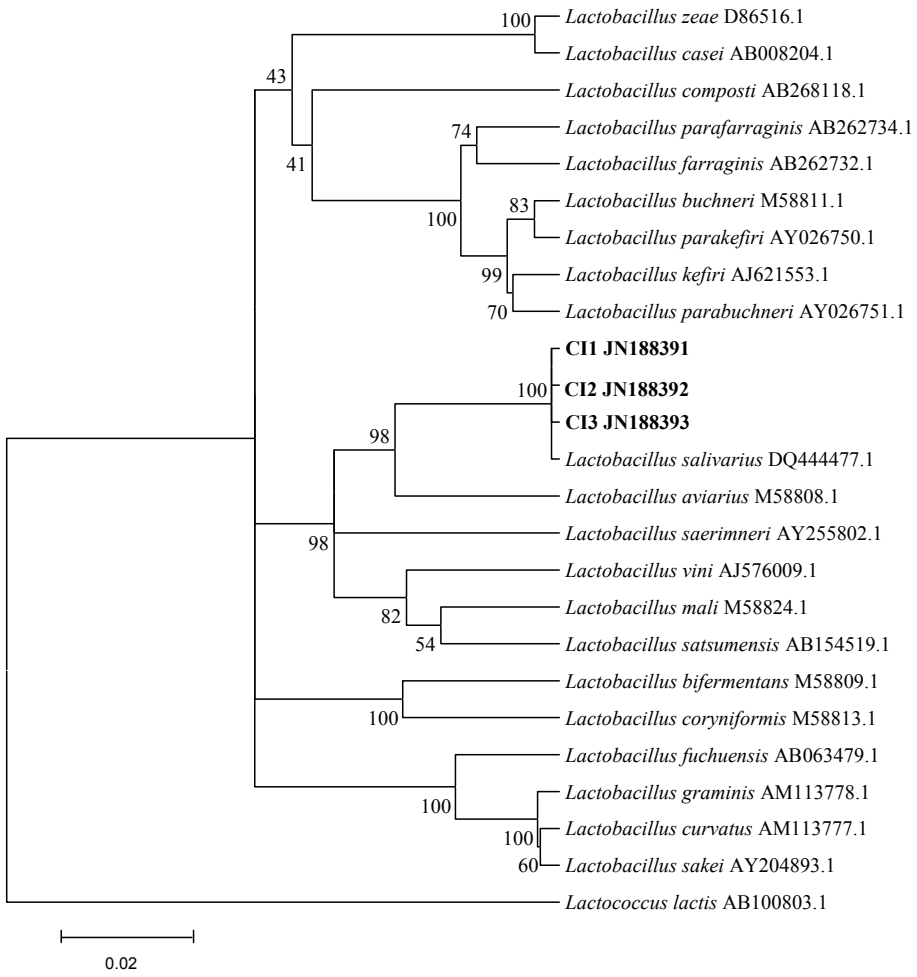


Fig.1: Phylogenetic tree based on 16S rRNA gene sequence analysis depicting the phylogenetic relationships among species of the genus *Lactobacillus* using the Neighbor-Joining method (Saitou and Nei, 1987). The outgroup was *Lactococcus lactis* (AB100803.1). The analysis involved 18 nucleotide sequences including three sequences of strains obtained in this study and 14 sequences belong to Lactobacillus species obtained from the GenBank (NCBI). Bootstrap values above 50% are indicated at the nodes of the tree. The scale bar represents 0.02-nucleotide substitutes per position.

significantly ($P < 0.05$) lower reduction in cell viability than *L. salivarius* CI2 and the reference strain *L. reuteri* C10, with 0.44 and 0.47 log units reduction in cell viability, respectively, which indicated a higher tolerance of *L. salivarius* CI1 and CI3 to the acidic condition than *L. salivarius* CI2 and the reference strain *L. reuteri* C10. Ehrmann *et al.* (2002) also found the strains of *L. reuteri*, *L. salivarius* and *L. animalis* to be tolerant to pH 3 for 4 h. Earlier, Charteris *et al.* (1998) reported in their review that most *Lactobacillus* spp were able to tolerate pH 4 for 1 h but the percentage of cell viability varied considerably among different strains.

In the chicken intestine, the total bile salt concentrations in the duodenum and cecum have been reported to be 0.175 and 0.008%, respectively (Lin *et al.,* 2003). In many studies, however, the standard level of 0.3% bile was considered for investigation of bile tolerance of potential probiotic *Lactobacillus* strains (Gilliland *et al.,* 1984; Jacobsen *et al.,* 1999; Boonkumklao *et al.,* 2006; Koll *et al.,* 2008; Ruiz-Moyano *et al.,* 2008; Sahadeva *et al.,* 2011). Thus, in the present study, 0.3% bile concentration was used. The results of the bile tolerance assay (Table 4) showed that all three *L. salivarius* strains exhibited bile tolerance (reduction in cell viability of 1.43 to 1.69 log units) at this concentration of bile salt, however, their tolerance levels were lower ($P < 0.05$) than that of the reference strain *L. reuteri* C10 (reduction in cell viability of 0.46 log units). A similar finding was reported by Koll *et al.* (2008) who found that all 67 *Lactobacillus* strains tested for their bile tolerance at 0.3% bile exhibited tolerance. Jin *et al.* (1998) also found that all 12 *Lactobacillus* strains studied were able to tolerate 0.3% of bile salt, while Jacobsen *et al.* (1999) reported that 41 of 42 tested *Lactobacillus* strains could tolerate bile at this concentration.

Pancreatic enzymes are secreted into the small intestine through the pancreatic duct and they are involved in digestion of proteins, carbohydrates, and fats in foods. As such, some studies have included the ability to tolerate the presence of pancreatic enzymes as another criterion for selection of probiotic cultures (Salminen, 1998; Ronka *et al.,* 2003). In this study, 3 h of exposure to pancreatic enzymes had little

TABLE 3
Viability of *Lactobacillus* strains after 3 h exposure to pH 3 and pH 7.2 (control)

| *Lactobacillus* strain | Cell viability (log CFU/ml)[1] | | Reduction in cell viability (log units)[1] |
|---|---|---|---|
| | pH 7.2 | pH 3 | |
| *L. reuteri* C10* | 7.50±0.07 | 7.03±0.06 | 0.47 [a] |
| *L. salivarius* CI1 | 7.26±0.02 | 7.21±0.01 | 0.05 [b] |
| *L. salivarius* CI2 | 7.94±0.05 | 7.50±0.09 | 0.44 [a] |
| *L. salivarius* CI3 | 7.30±0.04 | 7.25±0.02 | 0.05 [b] |

[1] Values are means ± SD of two independent experiments, each in triplicate
[a – b] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

adverse effect on the survival of the three *L. salivarius* strains (Table 5). All the three *L. salivarius* strains showed a very good tolerance to pancreatic enzymes (reduction in cell viability of 0.01 to 0.21 log units) and their tolerance levels were significantly ($P < 0.05$) higher than that of the commercial reference strain, *L. reuteri* C10 (reduction of cell viability of 0.46 log units). A similar result was reported by Ronka *et al*. (2003) who found that 3 h of incubation in growth medium containing pancreatic enzymes had little effect on viability of *L. brevis* strains. Ruiz-Moyano *et al.* (2008) also reported that 46 out of 51 tested lactic acid bacterial strains survived after 3 h of treating with 1.9 mg/ml of pancreatic enzymes.

*Adherence Ability*

Every potential probiotic strain is expected to attach to the epithelial cells of the intestine in order to colonize and establish in the intestine (Lee & Salminen, 1995). Furthermore, strong adherence to the intestine is necessary for releasing some probiotic bio-effects such as cholesterol lowering effects (Marteau, 2002), immune-modulation (Schiffrin *et al.,* 1995), and antimicrobial activities against pathogens (Mack *et al.,* 1999). In the present assay, adherence of the three *L. salivarius* strains to the Caco-2 cell line was in the range of 10 to 15 cells per Caco-2 cell (Table 6). *Lactobacillus salivarius* CI2 with an

TABLE 4
Growth of *Lactobacillus* strains in MRS broth (control) and MRS broth containing 0.3% bile salt

| *Lactobacillus* strain | Cell viability (log CFU/ml)[1] | | Reduction in cell viability (log units)[1] |
|---|---|---|---|
| | MRS | MRS + 0.3% bile salt | |
| *L. reuteri* C10* | 8.28±0.03 | 7.82±0.06 | 0.46 [a] |
| *L. salivarius* CI1 | 8.24±0.03 | 6.55±0.37 | 1.69 [b] |
| *L. salivarius* CI2 | 8.24±0.03 | 6.81±0.30 | 1.43 [b] |
| *L. salivarius* CI3 | 8.28±0.09 | 6.67±0.41 | 1.61 [b] |

[1] Values are means of two independent experiments, each in triplicate
[a – b] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

TABLE 5
Viability of *Lactobacillus* strains after 3 h exposure to 1.9 mg/ml pancreatic enzymes and normal condition (control)

| *Lactobacillus* strain | Cell viability (log CFU/ml)[1] | | Reduction in cell viability (log units)[1] |
|---|---|---|---|
| | Control | 1.9 mg/ml pancreatic enzymes | |
| *L. reuteri* C10* | 7.59±0.05 | 7.14±0.07 | 0.45 [a] |
| *L. salivarius* CI1 | 6.94±0.04 | 6.73±0.08 | 0.21 [b] |
| *L. salivarius* CI2 | 7.77±0.06 | 7.68±0.06 | 0.09 [c] |
| *L. salivarius* CI3 | 6.87±0.04 | 6.86±0.04 | 0.01 [c] |

[1] Values are means of two independent experiments, each in triplicate
[a – c] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

TABLE 6
Adherence of cells of Lactobacillus strains to Caco-2 cell

| *Lactobacillus* strain | Adhesion index (Lactobacillus cells per Caco-2 cell)[1] |
|---|---|
| *L. reuteri* C10* | 10.5±0.1 [c] |
| *L. salivarius* CI1 | 13.1±0.5 [b] |
| *L. salivarius* CI2 | 15.3±0.4 [a] |
| *L. salivarius* CI3 | 10.3±0.0 [c] |

[1] Values are means of two independent experiments, each in triplicate
Adherence was evaluated in 20 random microscopic fields
[a – c] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

adherence ability of 15 cells per Caco-2 cell and *L. salivarius* CI1 with an adherence ability of 13 cells per Caco-2 cell showed significantly ($P < 0.05$) higher adherence ability than *L. salivarius* CI3 and the commercial reference strain *L. reuteri* C10, both with an adherence ability of 10 cells per Caco-2 cell. Similar findings were reported by Jacobsen *et al.* (1999) who studied 47 *Lactobacillus* strains for their ability to adhere to Caco-2 cells and found considerable variations, from strong to low adhesion, among the strains. Gopal *et al.* (2001) also found that *L. rhamnosus* DR20, *L. acidophilus* HN017 and *B. lactis* DR10 exhibited strong ability to adhere to the Caco-2 and HT-29 human epithelial cell lines.

## CONCLUSION

Three bacterial strains isolated from the intestines of chickens were identified as *L. salivarius* using carbohydrate fermentation profile analysis by the API system and 16S rRNA gene sequencing. *In vitro* assays showed that all three *L. salivarius* strains exhibited good acid, bile and pancreatic enzyme tolerance, and good ability to adhere to Caco-2 cells. This indicated that all three *L. salivarius* strains would probably be able to survive, attach, and colonize GIT of chickens and could be considered as potential probiotic candidates for chickens. However, further *in vivo* studies in chickens need to be undertaken to evaluate the efficacy of the three *L. salivarius* strains in host animals.

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