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In Vitro Selection of Potential Lactic Acid Bacteria Isolated from Ducks and Geese in Thailand

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ABSTRACT

Potential lactic acid bacteria from the feces of ducks and geese in Thailand were selected and their probiotic properties were characterized for animal probiotic supplementation. A total of 104 isolates were subjected to an inhibitory activity test against pathogenic bacteria. The 101 isolates showed varying inhibition zones depending on the target pathogens. Tests for acid and bile tolerance were performed to test the bacterial survival. The results showed that 28 isolates survived in de Man Rogosa Sharp (MRS) medium mixed with 3% fresh chicken bile and in MRS medium at pH levels of 2, 3 and 4. A test of their adhesion ability to host epithelial cells using cell surface hydrophobicity produced a range of 2.72%–27.70%. The isolates were tested to determine their antimicrobial susceptibility profiles with gentamycin, ciprofloxacin, ampicillin, erythromycin, vancomycin and tetracycline. According to the criteria for *in vitro* probiotics selection, 3 of the 28 lactic acid bacteria isolates showed suitable probiotic properties. The bacteria were identified using phenotypic and genotypic methods. Based on 16S rRNA gene sequences analysis, strains D5-2 and D6-4 were classified as *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC11842^T with 99.00% identities, while strain D2-2-1 with 96.62% identities was closely related to *L. reuteri* F275^T.

Keywords: ducks, feces, geese, lactic acid bacteria, probiotics

INTRODUCTION

In the complex and diverse microbial community comprising hundreds of bacterial species in an animal's gastrointestinal tract (GIT), lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria (Collin *et al.*, 1991). Due to the ban on growth-promoting antibiotics in European Union countries, the uses of probiotics to improve growth performance and inhibit pathogenic bacteria in animals have been widely studied (Chang *et al.*, 2001; Hopkins and Macfarlane, 2002).

When LAB adhere to the host epithelial cells, they promote a beneficial microbial-host relationship by producing several antimicrobial compounds including organic acids, hydrogen peroxide and bacteriocin to reduce the numbers of pathogenic bacteria (Dunne *et al.*, 1999). These functions result in the improvement of feed conversion ratios, tolerance to diseases and an increase in egg production (Hyrominus *et al.*,

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2000; Ahmad, 2006).

According to a report of a joint international working group on drafting guidelines for the evaluation of probiotics in food (Food and Agriculture Organization/World Health Organization, 2002), the basic selection criteria for LAB strains to be used as probiotics have been described as follows: they should be tolerant to acid and bile, be able to adhere to the GIT epithelial cells and have the ability to produce antimicrobial substances for antagonistic activity against pathogenic bacteria. *In vivo* studies investigating the health benefits of potential LAB are time consuming and often expensive and for this reason, the use of *in vitro* tests as selection criteria to screen the most effective organisms are recommended (Taheri *et al.,* 2009b).

The selection and screening processes of the probiotic properties of LAB isolated from birds in Thailand were extensively studied regarding the isolation and characterization of the LAB isolated from chicken GIT (Nitisinprasert *et al.,* 2000; Musikasang *et al.,* 2009) but the study of other domestic poultry in Thailand is still limited. Therefore, the aims of this study were: 1) to screen and identify LAB isolated from the feces of grazing ducks and geese; 2) evaluate their probiotic effects based on *in vitro* tests of inhibitory activity against pathogenic bacteria, acid and bile tolerance, their adhering ability to host epithelial cells; and 3) to determine their antimicrobial susceptibility profiles.

MATERIALS AND METHODS

LAB isolates and growth conditions

LAB were isolated from feces samples of 38 healthy grazing ducks and 4 geese in 4 local family farms located in Nakhon Pathom, Chachoengsao and Nakhon Ratchasima during September and November 2011. The animals were aged around 1–2 yr. The feces were collected using sterilized cotton swabs and cultured in de Man Rogosa Sharp (MRS) broth (Difco™; Pessac, France) at pH 5.5. They were kept in ice boxes at 4 °C and delivered to the laboratory within 1–2 hr. The isolation procedure was modified from Hiraga *et al.* (2008). Samples were incubated at 37 °C for 48 hr. The cell suspensions were subcultured onto MRS agar containing 0.5% calcium carbonate ($CaCO_3$) at 37 °C for 48 hr under anaerobic conditions generated by Anaeropack®-Anaero (Mitsubishi Gas Chemical Company Inc.; Tokyo, Japan). Single colonies that presented a clear zone surrounding the colony were Gram stained for microscopic examination and tested for catalase production.

Detection of inhibitory activity

The inhibitory activity of each LAB sample was tested against pathogenic bacteria by agar well diffusion assay following the procedure described by Kizerwetter-Swida and Binek (2005). LAB were grown in MRS broth at 37 °C for 48 hr. Cells were removed by centrifugation at 13,000×*g* for 5 min and the supernatant was filtered through 0.20 µm pore-sized membrane (Minisart®, Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Staphylococcus aureus ATCC25923, *Escherichia coli* ATCC25922 and pathogens derived from the clinical samples of the Diagnostic Unit, Faculty of Veterinary Medicine, Kasetsart University, Kampaengsaen campus (*S. aureus*; vancomycin-resistant *Enterococcus faecium*; amoxicillin-, ampicillin- and oxytetracycline-resistant *Salmonella* Typhimurium; and colistin-resistant *E.coli*) were chosen for the study of the inhibitory activity of the LAB.

The pathogenic bacteria were inoculated in a nutrient broth and adjusted to match McFarland no.0.5 standard and then streaked onto nutrient agar. Wells were made using a cork borer and 50 µL of LAB suspension was added. The plates were incubated at 37 °C for 48 hr and the diameter of growth inhibition around each well was measured; inhibition zones of 8 mm or more were scored as positive.

Acid and bile tolerance test

In vitro GIT conditions were simulated for acid and bile by adjusting the MRS broth to pH 2, 3, 4 with 2M hydrochloric acid and fresh chicken bile at 3%, respectively. LAB isolates were inoculated into the adjusted MRS broth for 0 and 3 hr at 37 ºC, and the viable cell count was determined by dilution plating on MRS agar after incubation at 37 ºC for 48 hr (Jin *et al*., 1998). Colony forming unit (cfu) counts were calculated for the survival percentages using Equation 1:

$$\% \text{ survial} = \frac{\text{3 hr survival count of LAB (log cfu.mL}^{-1}\text{)}}{\text{Initial count of LAB (log cfu.mL}^{-1}\text{)}} \times 100 \quad (1)$$

Cell surface hydrophobicity test

The bacterial adhesion assay was modified from Kaushik *et al.* (2009) to measure the cell surface hydrophobicity. LAB cells grown in MRS broth at 37 ºC for 48 hr were centrifuged and the cell pellets were washed twice with phosphate buffer saline (PBS) at pH 7.4. The pellets were resuspended in PBS with absorbance (Abs) adjusted to approximately 0.7 optical density (OD) at 600 nm. Then, 750 µL of LAB cell suspension and 250 µL of n-hexadecane were mixed and incubated at 37 ºC for 10 min. The mixture was then agitated again and incubated at 37 ºC for 1 hr for phase separations. The aqueous phase was gently removed to measure its absorbance at 600 nm. The percentage of surface hydrophobicity was calculated as the OD decrease in the aqueous phase using Equation 2:

$$\% \text{ surface hydrophobicity} = 100 \times \frac{[\text{Abs (before mixing)} - \text{Abs (after mixing)}]}{\text{Abs (before mixing)}} \quad (2)$$

Antimicrobial susceptibility test

An antimicrobial susceptibility test was determined by modifying the procedures of Clinical and Laboratory Standards Institute (2011) and Klare *et al*. (2005). The test was done on Mueller-Hinton medium supplemented with 10% MRS medium under anaerobic conditions at 37 ºC for 48 hr. Antimicrobial disks (Oxoid GmbH, Wesel, Germany) were composed of gentamycin (120 µg), ciprofloxacin (15µg), ampicillin (10 µg), erythromycin (15 µg), vancomycin (30 µg) and tetracycline (30 µg).

Species identification

The selected LAB isolates were subjected for species identification by phenotypic and genotypic methods. The phenotype was characterized with biochemical identification using the analytical profile index (API) system (bioMèrieux Inc.; Marcy l'Etoile, France). A single colony on MRS agar was picked and suspended in API 50 CHL media (bioMèrieux Inc.; Marcy l'Etoile, France) and incubated at 37 ºC for 48 h. Sugar utilization was examined and the results were registered for identification using the Apiweb Software, Version 5.1 (bioMèrieux Inc.; Marcy l'Etoile, France).

Genomic DNA samples of the selected LAB were extracted using a DNA extracting kit (Invitrogen™; Life Technologies Corp.; Grand Island, NY, USA) and used as DNA templates. The 16S ribosomal RNA (16S rRNA) genes were amplified using 16S rRNA universal primers, 8-27f (5'- AGAGTTTGATCATGGCTCAG-3') and 1492r (5'-TACGGCTACCTTGTTACGACTT-3'). Each reaction contained 10× Ex Taq buffer, 10 mM of deoxynucleotide triphosphates, 40 µM of each primer and 5 U of Takara Ex Taq™ (Takara Bio Inc., Shiga, Japan). The polymerase chain reactions were carried out in the thermal cycle (GeneAmp®, PCR System 9700; Applied Biosystems; Singapore) by cyclic profile programming, predenaturation (10 min at 95 ºC) followed by 30 cycles of denaturation (1 min at 94 ºC), annealing (1 min at 55 ºC), extension (1.50 min at 72 ºC) and final extension (7 min at 72 ºC). Gene sequencing was carried out by First Base Laboratories, Selangor, Malaysia. The sequenced data were analyzed using BLAST server, BioEdit version 7.0.0 (Hall, 1999) and CLUSTAL_X version 1.83 (Thompson *et al.*,

1997). A phylogenetic tree was constructed based on the neighbor-joining method (Saitou and Nei, 1987) by the NJPlot program version 2.3 (Perrière and Gouy, 1996).

RESULTS

The probiotic characterizations of the LAB were studied using *in vitro* procedures following the guidelines of Food and Agriculture Organization/World Health Organization (2002). In total, 104 suspected LAB were isolated from 42 fecal samples. All of the 104 isolates were Gram positive and catalase negative. Of the 104 isolates, 100 isolates were bacilli and 4 isolates (C3-3, C10-5, E9-1 and E10-2) were cocci.

Detection of inhibitory activity

Cell free supernatant of the 101 isolates presented varying inhibition zones except for three isolates which did not show antimicrobial activity toward any of the bacterial pathogens. Of the 101 LAB, 75 isolates exhibited inhibitory activity against all six pathogens, while 26 isolates showed antagonistic activity against 2 to 5 bacterial pathogens.

Acid and bile tolerance test

The 101 inhibiting positive isolates were selected for the acid and bile tolerance test to investigate the survival rates by simulating the stress conditions found in the avian GIT. The results showed that 55 isolates and 54 isolates survived at pH 3 and pH 4, respectively. Under the strong acidic condition of pH 2, 28 LAB isolates survived with the survival rate ranging from 2.00 to 75.66%. Under the basic condition (3% fresh chicken bile), 51 isolates survived.

Antimicrobial Susceptibility test

The results of the antimicrobial susceptibility profiles among the 28 LAB showed that 17.86% (five isolates) of the isolates were resistant to all of the antimicrobials used in this study and 92.86% (26 isolates) of the isolates were resistant to 30 µg vancomycin, with the two susceptible isolates being D2-2- 1 (Table 1) and D2-8-1.

Table 1 Adhesion ability, antimicrobial susceptibilities profiles and 16S rRNA gene sequences analysis of the three resilient lactic acid bacteria.

Characteristic	D2-2-1	D5-2	D6-4
Source	Duck	Goose	Goose
Adhesion ability[a]			
% hydrophobicity	27.70 ± 0.07	26.00 ± 0.25	26.12 ± 0.25
Antimicrobial susceptibility profiles[b]			
Gentamycin (120 µg)	20.00 (S)	32.00 (S)	32.00 (S)
Ciprofloxacin (5 µg)	0.00 (R)	13.00 (R)	12.00 (R)
Ampicillin (10 µg)	31.00 (S)	31.00 (R)	30.00 (R)
Erythromycin (15 µg)	34.00 (S)	32.00 (S)	31.00 (S)
Vancomycin (30 µg)	27.00 (S)	0.00 (R)	0.00 (R)
Tetracycline (30 µg)	31.00 (S)	32.00 (S)	32.00 (S)
16S rRNA sequences analysis (% identities)[c]	*L. reuteri* F275^T (96.62%)	*L. delbrueckii* subsp. *bulgaricus* ATCC11842^T (99.00%)	*L. delbrueckii* subsp. *bulgaricus* ATCC11842^T (99.00%)

[a] = Adhesion ability of isolates using hydrophobicity test (% hydrophobicity), values are shown as mean ± SD.

[b] = Zone diameter (mm) of antimicrobial susceptibility test: (S) = Susceptible, (I) = Intermediate, (R) = Resistant.

[c] = Species identification based on 16S rRNA genes.

Cell surface hydrophobicity test

The results of the cell surface hydrophobicity test showed 28 isolates with different percentages of hydrophobicity ranging from 2.72% to 27.70%. Isolates D2-2-1, D5-2 and D6-4 exhibited the top three percentages of hydrophobicity of 27.70, 26.00 and 26.12%, respectively (Table 1).

Species identification

Finally, the 3 LAB isolates (D2-2-1, D5-2 and D6-4) that exhibited the greatest percentages of adhesion ability and presented suitable probiotic properties including inhibitory activity and tolerance to GIT stressing conditions (Table 2) were selected for further study of their biochemical characteristics and 16S rRNA genes.

Based on biochemical analysis, the isolates D5-2 and D6-4 from geese were determined to be *Lactobacillus acidophilus* with 72.70% identification by API 50 CHL (bioMèrieux Inc.; Marcy l'Etoile, France). The isolate D2-2-1, originally isolated from ducks, was determined as *L. fermentum* with 53.60% identities.

Based on 16S rDNA sequences analysis, the isolates D5-2 (accession number KC574073) and D6-4 (accession number KC574074) were identified at 99.00% to be *L. delbrueckii* subsp. *bulgaricus* ATCC11842^T. The bacterial number D2-2-1 (accession number JQ776750) was identified as being closely related to *L. reuteri* F275^T (96.62% identities). A phylogenetic tree of the three LAB isolates was constructed (Figure 1). The isolates D5-2 and D6-4 were closely related to *L. delbrueckii* subsp. *bulgaricus* ATCC11842^T. The isolate D2-2-1 was closely related to *L. reuteri* F275^T and represented a separate species.

DISCUSSION

The probiotic characterizations of the LAB were studied using *in vitro* procedures following the guidelines for the evaluation of probiotics in food (Food and Agriculture

Table 2 Inhibitory activity of the three resilient lactic acid bacteria (LAB) against pathogenic bacteria and their survival percentages under simulated gastrointestinal tract stressing conditions.

LAB isolate	Inhibitory activity (mm)						% Survival in acid and bile conditions			
	E.coli ATCC25922	*S.aureus* ATCC25923	*E.faecium*	*S.*Typhimurium	*E.coli*	*S.aureus*	pH 2	pH 3	pH 4	3% bile
D2-2-1	24.50 ± 0.71	15.00 ± 0.00	16.00 ± 0.00	12.50 ± 0.71	15.50 ± 0.71	15.50 ± 0.71	65.14 ± 0.01	77.10 ± 0.07	89.45 ± 0.07	66.40 ± 0.14
D5-2	12.00 ± 0.00	11.00 ± 0.00	11.00 ± 0.00	11.50 ± 0.71	11.00 ± 0.00	11.50 ± 0.71	55.68 ± 0.21	72.00 ± 0.48	79.13 ± 0.06	41.00 ± 2.01
D6-4	12.00 ± 0.00	10.50 ± 0.71	11.00 ± 0.00	12.00 ± 0.00	11.00 ± 0.00	11.00 ± 0.00	56.37 ± 1.09	73.08 ± 2.87	78.32 ± 0.69	42.16 ± 0.38

Values are shown as mean ± SD.

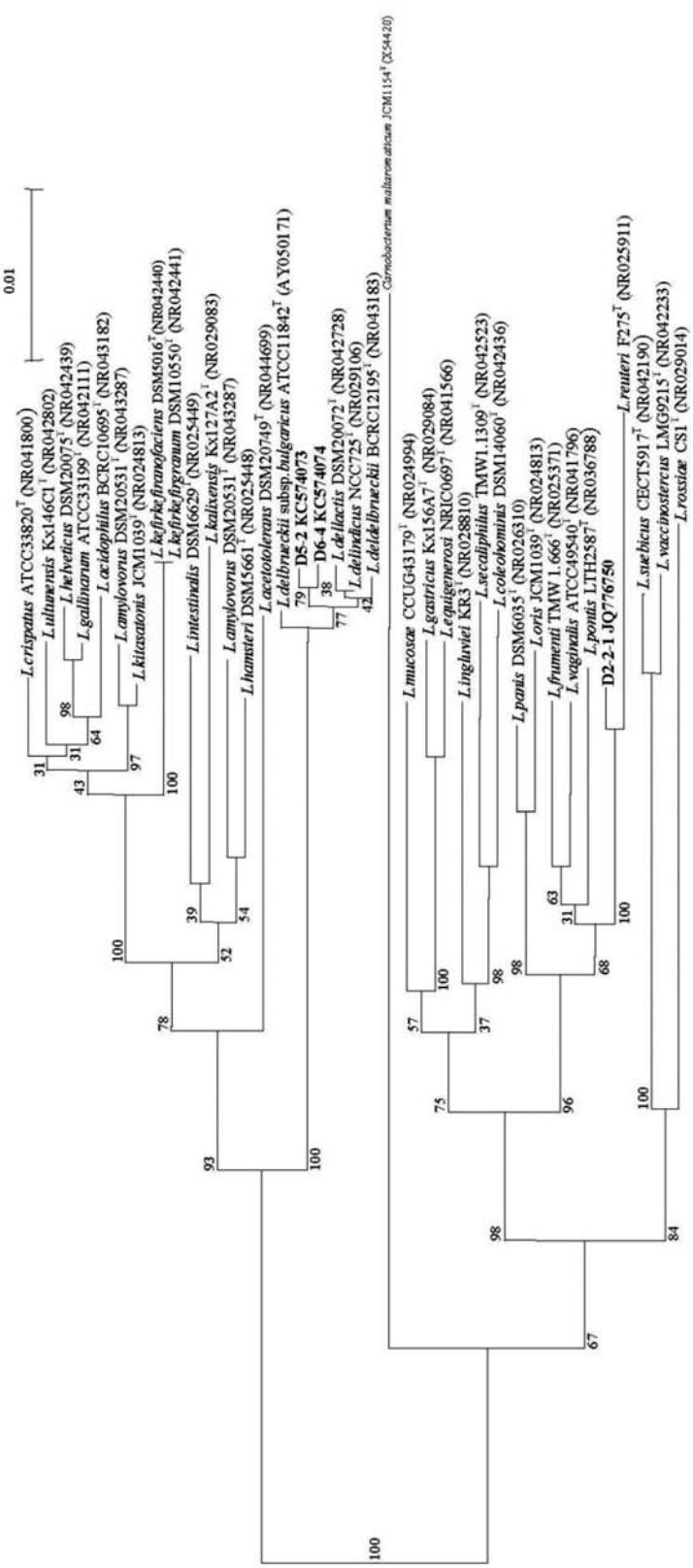

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolates D2-2-1, D5-2, D6-4 and selected lactobacilli. The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 55 %, based on 1,000 replications, are shown at nodes. The bar scale represents 0.01 substitutions per nucleotide position.

Organization/World Health Organization, 2002). For inhibitory activity against bacterial pathogens of the isolates, the supernatant of each of the 104 isolates was not neutralized. Therefore, the mode of inhibition is not known exactly. The inhibitory activity of some LAB may have been due to antimicrobial compounds other than lactic acid. Additional investigations should consider adjusting the pH of the supernatants of all inhibiting positive isolates to around 7 (Todorov and Dicks, 2005) to determine their inhibitory activities toward bacterial pathogens due to bacteriocins and the typical characteristics of bacteriocins should be determined also.

Before being able to colonize the GIT epithelial cells, LAB must first survive the transit through the upper GIT where the acidity can be as low as 2 to 4 pH (Denbow, 2000). Tolerance to bile is considered to be essential for the colonization and metabolic activity of bacteria in the GIT (Havenaar *et al.,* 1992). Some LAB strains could produce bile salts hydrolase, the specific enzyme which enables the hydrolyzation of conjugated bile, thus reducing the toxic effects of bile (Du Toit *et al.,* 1998). In comparison to other studies, the medium used in the current study (pH 2 and 3% fresh chicken bile) simulated more extreme conditions (Taheri *et al.,* 2009b; Nouri *et al.,* 2010). However 28 LAB isolates still survived.

A disc diffusion method was performed to screen the antimicrobial susceptibility profiles of the isolates. It is well known that vancomycin is a glycopeptide antimicrobial that inhibits the peptidoglycan synthesis which is an important structural component of cell walls in Gram-positive bacteria (Sinha and Neuhaus, 1968). LAB are especially vulnerable to vancomycin treatment (Reynolds, 1989). The present results agree with those reported by Danielsen and Wind (2003) that there is a high natural resistance to vancomycin in all lactobacilli. However, there are no generally accepted standard procedures for antimicrobial determination for LAB. A major problem in proposing a breakpoint of antimicrobial susceptibilities for LAB is the lack of relevant data (Mathur and Singh, 2005). The present study applied the zone diameter standard that is commonly used for interpretation of *Enterococcus* spp. susceptibility (Clinical and Laboratory Standards Institute, 2011) because the genus *Enterococcus* is one of the LAB that inhabit animal GIT.

To determine the adhesion ability of the isolates, bacterial cell surface hydrophobicity could be applied to an indirect examination of the adhesion ability to host epithelial cells (Taheri *et al.,* 2009a). As the hydrocarbon has a strong relationship to the epithelium of GIT, high percentages of surface hydrophobicity indicated that bacteria have more chance to adhere to host epithelial cells (Garriga *et al.,* 1998) and are able to survive and maintain their health-promoting properties.

Three LAB isolates (D2-2-1, D5-2 and D6-4) that exhibited the highest activity of adhesion ability and presented suitable probiotic properties were also grown on MRS media for 48 hr at 42 ^{o}C which is the temperature of an avian's body. They were subjected for species identification using genotypic and phenotypic methods. All of the three resilient LAB isolates were originally isolated from ducks and geese raised on one family farm in Pak Chong district, Nakhon Ratchasima province, Thailand. The isolates D5-2 and D6-4 were found in the feces of different individual geese. The isolate D2-2-1 was isolated from duck feces. The animals on this farm were not confined and were allowed to scavenge for food over a wide area, feeding on such resources as snails, plant grains and aquatic plants in ponds. Moreover, the flock contained different species of birds with varying ages and they were raised in combination with wild boars. Such an environment was suspected to provide a greater diversity of bacteria especially in the GIT of the grazing ducks and geese.

In this study, 16S rRNA gene sequence analysis was chosen as the means of identifying

the bacteria. After the phylogenetic tree was generated, the isolates D5-2 and D6-4 were closely related to *Lactobacillus delbrukii* subsp. *bulgaricus* ATCC11842^T. The isolate D2-2-1 was closely related to *Lactobacillus reuteri* F275^T at 96.62% identities. This value is lower than 97% which is the recommended value for species identification (Stackebrandt and Goebel, 1994). Therefore, it could be further identified using other assays, for example multilocus sequence analysis of housekeeping genes, or DNA-DNA hybridization, to confirm the result better than by using only a part of the nucleotide sequence of 16S rDNA. (Berger *et al.*, 2007).

L. delbrueckii subsp. *bulgaricus* was reported by Simova *et al.* (2008) as a strain isolated from Bulgarian yoghurt and safe for probiotics supplementation. To the knowledge of the authors of the present study, this present study is the first to report the isolation of *L. delbrueckii* subsp. *bulgaricus* in geese feces. Additionally, in other studies, *L.reuteri* isolated from chicken intestine displayed a broad inhibition spectrum against *E. coli*, *Salmonella* spp. (Nitisinprasert *et al.*, 2000) and *Campylobacter* spp. (Nazef *et al.*, 2008).

CONCLUSION

According to the *in vitro* tests used in this study, three resilient LAB isolated from ducks and geese feces were considered as potential probiotic candidates for further investigation to be used as a probiotic feed supplement in the poultry industry.

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