# ความไวต่อยาต้านจุลชีพของเชื้อแลคโตบาซิลไลโปรไบโอติกที่แยกได้จากมูลไก่

# Antimicrobial Susceptibility of Probiotic Lactobacilli Isolated from Chicken Feces

*ไพรัตน์ ศรแผลง (Pairat Sornplang)*[1*]
*วิชัย ลีลาวัชรมาศ (Vichai Leelavatcharamas)*[2]

## บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติเบื้องต้นและรูปแบบความไวต่อยาต้านจุลชีพของแบคทีเรียแลคโตบาซิลไลโปรไบโอติกที่แยกได้จากมูลไก่พื้นเมือง เชื้อแลคโตบาซิลลัสที่แยกได้ทั้ง 65 ไอโซเลท ถูกนำมาตรวจหาความสามารถในการทนกรด ทนเกลือน้ำดีและความไวต่อยาต้านจุลชีพ 11 ชนิด โดยการทดสอบความไวต่อยาต้านจุลชีพใช้วิธี disk diffusion จากนั้นนำเชื้อแลคโตบาซิลลัสที่คัดเลือกได้ที่มีความสามารถทนกรด ทนเกลือน้ำดีและไม่ต้านต่อยาต้านจุลชีพแบบได้รับมาภายหลัง (acquired resistance) มาแยกสายพันธุ์และจำแนกชนิด (species) ของเชื้อด้วยวิธี pulsed-field gel electrophoresis (PFGE) และการวิเคราะห์ลำดับนิวคลิโอไทด์ของจีน 16S rRNA ตามลำดับ ผลการทดลองพบว่ามีเชื้อแลคโตบาซิลลัส 11 ไอโซเลท ที่ทนต่อกรดและเกลือน้ำดี ส่วนรูปแบบความไวต่อยาต้านจุลชีพทั้ง 11 ชนิด พบว่าเชื้อแลคโตบาซิลลัสทั้ง 11 ไอโซเลท มี 6 ไอโซเลทที่มีความไวแบบมากต่อยาต้านจุลชีพ penicillin, tetracycline และ chloramphenicol และมีความไวแบบปานกลางต่อยาต้านจุลชีพ bacitracin, erythromycin และ nitrofurantoin และพบ 4 ไอโซเลท ดื้อต่อยาต้านจุลชีพ tetracycline และ 1 ไอโซเลทดื้อต่อยาต้านจุลชีพ bacitracin เชื้อแลคโตบาซิลลัสทั้ง 11 ไอโซเลทนี้ ดื้อต่อยาตามธรรมชาติ (intrinsic resistance) และต่อยาต้านจุลชีพ vancomycin, nalidixic acid และยากลุ่ม aminoglycoside เมื่ออาศัยคุณสมบัติการทนกรด การทนเกลือน้ำดีและการไม่ต้านต่อยาต้านจุลชีพแบบได้รับมาภายหลังจึงคัดเลือกแลคโตบาซิลลัสโปรไบโอติกได้ 6 ไอโซเลท และนำมาแยกสายพันธุ์ได้ 5 รูปแบบของ PFGE และเชื้อทั้ง 5 รูปแบบ PFGE นี้ถูกจำแนกเป็น *Lactobacillus salivarius* โดยมีความเหมือนของลำดับนิวคลิโอไทด์ที่ 99-100% เทียบกับ GenBank (accession number CP000233.1)

[1] *Assistant Professor, Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University*
[2] *Assistant Professor, Fermentation research center for value added agricultural products, Faculty of Technology, Khon Kaen University*
* *Corresponding author, e-mail: pirson@kku.ac.th*

# Abstract

This work aimed to study the primary properties and antimicrobial susceptibility patterns of probiotic lactobacilli isolated from native chicken feces. Sixty five isolates of *Lactobacillus* spp. were screened for acid and bile tolerant strains and also subjected to susceptibility test against 11 antibiotics using the disk diffusion method. All selected *Lactobacillus* isolates had acid and bile tolerant properties and none had acquired resistance to antibiotics. Antibiotics were distinguished and identified to species level using pulsed-field gel electrophoresis (PFGE) and nucleotide sequences analysis of the 16S rRNA gene, respectively. The results of acid and bile tolerance tests showed that 11 isolates exhibited positive signs. Six out of 11 isolates showed a strong sensitivity to penicillin, tetracycline and chloramphenicol and a moderate sensitivity to bacitracin, erythromycin and nitrofurantoin. Four isolates resisted tetracycline and 1 isolate had bacitracin resistance. All 11 isolates were resistant to vancomycin, nalidixic acid and aminoglycoside antibiotics (gentamicin, neomycin and streptomycin). Based on acid and bile tolerance and without acquired resistance to antibiotics, 6 probiotic lactobacilli were selected. They were differentiated into 5 different PFGE patterns. All isolates of 5 PFGE patterns were identified as *L. salivarius* with 99% to 100% nucleotide sequence identities compared to GenBank (accession number CP000233.1)


**คำสำคัญ:** ความไวต่อยาต้านจุลชีพ แลคโตบาซิลไล มูลไก่
**Keywords:** antimicrobial susceptibility, lactobacilli, chicken feces

## Introduction

Lactic acid bacteria (LAB) suitable for use as a probiotic must be tolerant to acid and bile under similar conditions to gastrointestinal environments and have no-transferable antibiotic resistance genes. Antibiotic resistance of LAB has two characteristics: (i) natural or intrinsic resistance, being non-transmissible; (ii) acquired resistance, usually caused from bacterial mutation or possibly carrying plasmid encoding of antibiotic resistance genes and potentially transmissible to other bacteria (Courvalin, 2006). The European Union requires LAB strain supplementation in animal feeds that have no antibiotic resistance genes of acquired resistance type. Antibiotic resistance of LAB is often intrinsic. However, some LAB isolated from animals may have an important role as an antibiotic resistance gene reservoir, due to many effects. These include antibiotic growth promoter used, overuse of antibiotic therapy in animals and closeness between human houses and animal rearing that can contaminate with antibiotic resistance genes of animal pathogens and potentially transmit to commensal or other pathogenic bacteria in the gastrointestinal tract (Mathur and Singh 2005). Therefore, the main objective of this study was investigation of antimicrobial susceptibility to eleven antibiotics, commonly used for antimicrobial susceptibility test by assessing the resistance phenotypes (European Food Safety Authority, 2008), of isolates of *Lactobacillus* spp. which were isolated from Thai native chicken feces.

## Materials and Methods

*Bacterial strains*

Sixty-five isolates of *Lactobacillus* spp. were obtained from the the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. They were stored in a modified freezing medium (Trypticase Soy Broth (Criterion™), 0.6% yeast extract and 20% glycerol) and kept at -70 °C. These lactic acid bacteria were isolated from Thai native chicken feces and identified into genus levels using physiological and biochemical characteristics according to Bergey's manual of systematic bacteriology and Salminen et al. (2004). These *Lactobacillus* isolates were successfully identified as described in our previous study (Sornplang et al., 2007).

*Acid tolerance test*

At first, all 65 *Lactobacillus* isolates were tested for acid tolerance. The acid tolerance of lactobacilli was studied according to Conway et al. (1987). Briefly, the cultures were grown in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, England) at 37 °C overnight, then sub-cultured into 10 ml of fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 4,000 rpm for 10 min, the pellets were washed twice in sterile phosphate buffered saline (PBS, pH 7.2; Sigma) and re-suspended in 1 ml of PBS (8.3 mmol/L). Each strain was diluted 1/100 in PBS at pH 3. Hydrochloric acid (1M HCl) was used to adjust the pH of the PBS. After 1, 2 and 3 h incubation, viable bacterial counts were determined by plating serial dilution (in Maximum Recovery Diluent, MRD; Oxoid, England) on MRS agar followed by incubation under microaerophilic (3.5% $CO_2$, 5% $O_2$, 7.5% $H_2$, 84% $N_2$) conditions (in anaerobic jar with GasPak® envelopes) at 37°C for 48 h. All tests were carried out in duplicate.

*Bile tolerance test*

All 65 *Lactobacillus* isolates were also tested for bile tolerance as described by Ehrmann et al. (2002) with some modifications. Briefly, the *Lactobacillus* strains were grown overnight in MRS broth and 1% of fresh culture ($10^5$ CFU) suspension was inoculated into tubes containing 10 ml of MRS broth with 1% (w/v) oxgall (Sigma, US). The inoculated tubes were incubated at 37 °C under microaerophilic conditions as described above and monitored hourly for 3 h by absorbance value at 600 nm ($O.D._{600}$) using a spectrophotometer. All tests were carried out in duplicate.

*Antimicrobial susceptibility test*

The selected acid- and bile- tolerant *Lactobacillus* isolates were further tested for antimicrobial susceptibility by the disk diffusion method (Bauer et al., 1966). Eleven antibiotics were chosen for the test: (i) β-Lactam group, inhibitors of cell wall synthesis: penicillin G (10 μg); (ii) gram-positive spectrum: erythromycin (10 μg), vancomycin (30 μg), bacitracin (10 μg); (iii) gram-negative spectrum: nalidixic acid (30 μg); (iv) broad spectrum: chloramphenicol (30 μg), tetracycline (30 μg); (v) inhibitors of protein synthesis: aminoglycosides—gentamicin (10 μg), neomycin (10 μg), streptomycin (10 μg); (vi) other broad spectrum: nitrofurantoin (300 μg). All antibiotic disks (diameter = 6 mm) were obtained from Oxoid (Oxoid, England).

The antimicrobial susceptibility test was similar to those as described by Charteris et al. (1998). Briefly, each *Lactobacillus* isolate was inoculated with $10^5$ CFU at 37 °C in MRS broth and incubated anaerobically for 18 h. Culture solution was dipped using sterile cotton swabs and swabbed in three directions on Mueller-Hinton agar plates. All antibiotic disks were seeded in the plates and incubated anaroebically at 37 °C for 48 h. The diameters of antibiotic inhibition zones

were measured using a ruler under a colony counter apparatus (Gallenkamp, England) and expressed in millimeters which included diameter of antibiotic disk. Antimicrobial susceptibility was interpreted according to the cut-off levels proposed by Charteris et al. (1998) with strains considered resistant if inhibition zone diameters were equal to or smaller than 19 mm for penicillinG, 14 mm for vancomycin and tetracycline, and 13 mm for kanamycin, chloramphenicol and erythromycin. Equal to or smaller than 10 mm and 13 mm of inhibition zone diameters for bacitracin and nalidixic acid were considered as resistant, respectively, according to the cut-off levels with minimal modifications of Baker et al. (1986). All antibiotics were tested in duplicate.

*Pulsed-field gel electrophoresis (PFGE) fingerprinting and 16S rRNA gene sequences*

The selected *Lactobacillus* isolates, which were acid and bile tolerant and had antimicrobial susceptibility, were differentiated using PFGE fingerprinting techniques. This technique followed the method of Karen et al. (2005) with some modifications. Briefly, 1.5 ml of log-phase of selected isolates at an optical density at 600 nm of 0.6 were used to prepare the agarose plugs, which were treated with proteinase K prior to the mutanolysin/lysozyme step to ensure cell lysis. Bacterial genomic DNAs of SmaI-digested and molecular weight markers were run in 1% of low melting point agarose gels (SeaKem agarose) in 0.5 M Tris-Borate-EDTA buffer (0.54 % Sigma 7-9, 0.05 % EDTA, 0.28% Boric acid, Milli Q water 100 ml, pH 8.0) at 14 $^{o}$C using a Bio-Rad CHEF-DR® III (Hercules, CA) under the following conditions: 6.0 V/cm, 3 s to 17.3 s switch time, linear ramping factor, 120$^{o}$ angle, and a run time of 19 h. Gels were analyzed visually, using the different bands of the isolates to determine the different strains. Based on different PFGE patterns of *Lactobacillus* isolates, they were identified at species level using 16S rRNA gene sequences. The region of 16S rRNA gene of lactobacilli DNA was amplified using primers SU forward (5′-CAC CAA CAG AGT TTG ATC CTG GCT CAG-3′) and HDA2 reverse (5′-GTA TTA CCG CGG CTG CTG GCA-3′) as described by Tannock et al. (2000). The primers were obtained from Professor Tannock's laboratory, Department of Microbiology and Immunology, University of Otago, New Zealand.

## Results

*Acid and bile tolerance test*

Eleven isolates (*Lactobacillus* L14, L,17, L31, L32, L33, L55, L56, L57, L61, L62 and L63 strains) of all *Lactobacillus* spp. (65 isolates) possessed acid and bile tolerance. The survival at pH 3 and in the presence of 1% oxgall of selected *Lactobacillus* isolates are shown in Table 1 and Table 2, respectively.

**Table 1.** Survival of the selected *Lactobacillus* isolates after incubation at pH 3

| Isolates | Survival (%) after incubation at pH 3 | | | |
|---|---|---|---|---|
| | 0 h | 1h | 2h | 3h |
| L14 | 100 | 89.5 | 80.5 | 80.1 |
| L17 | 100 | 88.8 | 80.4 | 80.1 |
| L31 | 100 | 89 | 80.3 | 80.1 |
| L32 | 100 | 90 | 81 | 80.5 |
| L33 | 100 | 89.7 | 81.1 | 80.3 |
| L55 | 100 | 90.5 | 84 | 82.5 |
| L56 | 100 | 87.9 | 80.2 | 80 |
| L57 | 100 | 88.6 | 81.2 | 80.4 |
| L61 | 100 | 100 | 100 | 93.5 |
| L62 | 100 | 89.6 | 80.6 | 80.2 |
| L63 | 100 | 89.5 | 80.7 | 80.3 |

**Table 2.** Effects of bile salt (1 % oxgall) on the growth of the selected *Lactobacillus* isolates

| Isolate[a] | OD 600 nm at various incubation times | | | | | |
|---|---|---|---|---|---|---|
| | Without oxgall | | | With oxgall | | |
| | 1 h | 2 h | 3 h | 1 h | 2 h | 3 h |
| L14 | 0.07 | 0.11 | 0.50 | 0.06 | 0.10 | 0.06 |
| L17 | 0.065 | 0.10 | 0.45 | 0.06 | 0.09 | 0.08 |
| L31 | 0.08 | 0.15 | 0.60 | 0.075 | 0.14 | 0.135 |
| L32 | 0.09 | 0.12 | 0.50 | 0.08 | 0.10 | 0.08 |
| L33 | 0.07 | 0.09 | 0.40 | 0.07 | 0.08 | 0.05 |
| L55 | 0.10 | 0.20 | 0.70 | 0.09 | 0.095 | 0.047 |
| L56 | 0.09 | 0.13 | 0.55 | 0.08 | 0.10 | 0.07 |
| L57 | 0.09 | 0.15 | 0.55 | 0.08 | 0.11 | 0.06 |
| L61 | 0.07 | 0.08 | 0.35 | 0.07 | 0.08 | 0.15 |
| L62 | 0.068 | 0.085 | 0.37 | 0.062 | 0.07 | 0 |
| L63 | 0.069 | 0.085 | 0.38 | 0.063 | 0.07 | 0 |

[a]Survival rate of selected isolates > 80% at 1% oxgall after 2 h incubation

*Antimicrobial susceptibility pattern of Lactobacillus isolates*

Results of antimicrobial susceptibility are shown in Table 3. All of 11 *Lactobacillus* isolates were susceptible to ß-Lactam antibiotic (penicillin) and broad spectrum antibiotic (chloramphenicol) and had moderate susceptibility to erythromycin and nitrofurantoin. All of the 11 *Lactobacillus* isolates were resistant to vancomycin, nalidixic acid, and aminoglycoside antibiotics (gentamicin, neomycin and streptomycin). Four out of 11 *Lactobacillus* isolates (L17, L33, L56 and L57) resisted tetracycline and 1 isolate (L32) showed resistance characteristics to bacitracin.

**Table 3.** Antimicrobial susceptibility of 11 acid and bile tolerant *Lactobacillus* isolates from Thai native chicken feces using the disk diffusion method.

| Isolate | Antimicrobial agent | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | P (10µg) | E (10µg) | VA (30µg) | B (10µg) | NA (30µg) | C (30µg) | TE (30µg) | CN (10µg) | N (10µg) | S (10µg) | F (300µg) |
| L14 | S(24)[1] | I (18) | R (7) | I (19) | R (8) | S (23) | S (23) | R (9) | R (7) | R (9) | I (19) |
| L17 | S (21) | I (17) | R (8) | I (18)[2] | R (9) | S (22) | R (13) | R (9) | R (7) | R (9) | I (18) |
| L31 | S (25) | I (19) | R (8) | I (19) | R (8) | S (24) | S (24) | R (8) | R (8) | R (9) | I (20) |
| L32 | S (21) | I (18) | R (9) | R (10) | R (9) | S (21) | S (21)[2] | R (9) | R (8) | R (10) | I (19) |
| L33 | S (22) | I (20) | R (9) | I (20) | R (10) | S (21) | R (12) | R (10) | R (9) | R (10) | I (20) |
| L55 | S (27) | I (20) | R (9) | I (20) | R (8) | S (26) | S (25) | R (10) | R (9) | R (11) | I (19) |
| L56 | S (22) | I (20) | R (7) | I (16) | R (8) | S (21) | R (14) | R (8) | R (8) | I (12) | I (18) |
| L57 | S (21) | I (19) | R (9) | I (18) | R (8) | S (21) | R (15) | R (10) | R (9) | R (11) | I (18) |
| L61 | S (29) | I (20) | R (8) | I (20) | R (8) | S (28) | S (27) | R (10) | R (8) | R (10) | I (20) |
| L62 | S (29) | I (20) | R (8) | I (20) | R (8) | S (28) | S (27) | R (10) | R (7) | R (10) | I (20) |
| L63 | S (28) | I (18) | R (9) | I (18) | R (9) | S (27) | S (26) | R (9) | R (9) | R (9) | I (18) |

[1]: Inhibition zone diameter in millimeters
[2]: showed pinpoint colonies within the inhibition zone
R: resistant; S: sensitive; I: intermediate
P=penicillin; E=erythromycin; VA=vancomycin; B=bacitracin; NA=nalidixic acid; C=chloramphenicol; TE=tetracycline; CN=gentamicin; N=neomycin; S= streptomycin; F=nitrofurantoin

*Pulsed-field gel electrophoresis (PFGE) fingerprinting and 16S rRNA gene sequences*

Six *Lactobacillus* isolates (L14, L31, L55, L61, L62 and L63) were selected for further identification, because they were acid and bile tolerance and susceptible to most antimicrobial agents, commonly used for antimicrobial susceptibility tests recommended by the European Food Safety Authority (European Food Safety Authority, 2008). These isolates were differentiated into 5 PFGE band patterns as shown in Figure 1. All 5 strains that had different PFGE band patterns were identified as *L. salivarius* using 16S rRNA gene sequences. They had high similarity (99-100%) compared to GenBank (accession number CP000233.1)

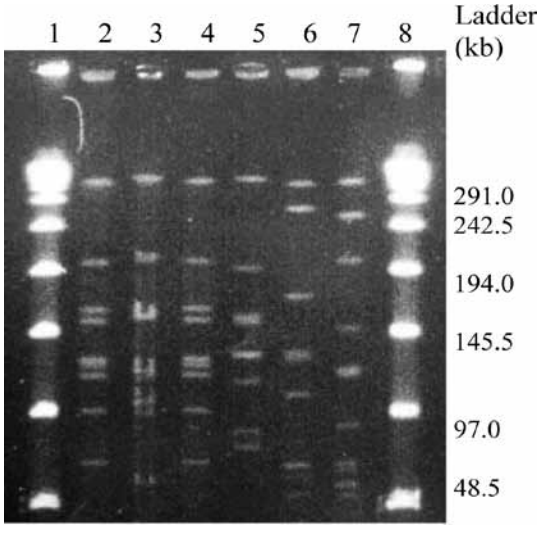


**Figure 1.** PFGE patterns of SmaI-digested genomic DNA from lactobacilli isolated from Thai native chicken faeces

**note:** Lane 2, L62 and Lane 4, L63 (PFGE group 1); Lane 3, L14 (PFGE group 2); Lane 5, L31 (PFGE group 3); Lane 6, L55 (PFGE group 4); Lane 7, L61 (PFGE group 5); Lane 1, 8, Lambda ladder PGE marker (New England Biolabs, Pickering, Ontario, Canada)

## Discussion

Eleven acid- and bile- tolerant *Lactobacillus* isolates in this study showed resistance to gram-negative spectrum antibiotic (nalidixic acid) and aminoglycoside antibiotics (gentamicin, neomycin and streptomycin). These results were similar to those of Zhou et al. (2005) and Termmerman et al. (2003) who reported that most *Lactobacillus*, *Enterococcus* and *Pediococcus* strains used as probiotics were resistant to gram-negative spectrum and aminoglycoside antibiotics. Resistance of these antibiotics is usually intrinsic; they are not transferring the genes to pathogenic or bacterial flora (Ammor et al., 2007). Vancomycin resistance was found in all isolates of *Lactobacillus* spp. These findings could be explained by concluding that vancomycin resistance was intrinsic for the genera *Lactobacillus* (Salminen et al., 1998). In contrast, the pathogenic bacteria such as *Escherichia coli* were usually susceptible to these antibiotics (Martin et al., 2005). This study showed that some *Lactobacillus* isolates had acquired resistance and could transfer the resistance gene to other bacteria. These isolates might have acquired the resistant gene from pathogenic bacteria due to animal feed supplements, such as growth promoters, when humans or pets consume antibiotic residue in meat or from antibiotic treatment in chicken of rural raisers in Thailand without veterinarian recommendation (Chalermchaikit et al., 2005). In addition, there were 2 out of 65 *Lactobacillus* isolates (L17 and L32) which showed pinpoint colonies within the inhibition zone with bacitracin and tetracycline, respectively. This observation for these bacterial strains indicates the mutation that leads to antimicrobial resistance (Danielsen and Wind 2003).

*Lactobacillus* species that are used as probiotics were isolated from various parts of chicken gut including crop, ileum, ceacum and feces. Some strains of L. *plantarum* and L. *fermentum*, isolated from ceacum and feces of chickens, showed resistance to pH 2 and 0.3% (w/v) oxgall (Shin et al., 2002) and had an adhesive property and *Salmonella* inhibition (Gusils et al., 2006). From our study, the 11 *Lactobacillus* species found in Thai native chicken feces resisted pH 3 and 1% (w/v) oxgall. Six out of 11 *Lactobacillus* isolates were safe under antimicrobial susceptibility tests. They were differentiated to 5 PFGE patterns. All of 5 PFGE patterns (L14, L31, L55, L61, L62 or L63) were identified as *Lactobacillus salivarius* using 16S rRNA gene sequences.

## Conclusions

In this study, 6 selected lactobacilli isolated from Thai native chicken feces showed 5 PFGE patterns as 5 different strains. They were inhibited by antimicrobial agents commonly used in therapy and prophylaxis in veterinary medicine. These lactobacilli could be guaranteed to be clear from antibiotic resistance genes. These isolates were intrinsically resistant (non-transferable) to nalidixic acid and aminoglycosides. All 5 selected isolates of *Lactobacillus* were intrinsically resistant to vancomycin. Therefore, these 5 *Lactobacillus* isolates are possible for selection as a probiotic in animals. All these isolates were identified as *Lactobacillus salivarius* using 16S rRNA gene sequences.

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