HIBISCUS PUBLISHER

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: https://journal.hibiscuspublisher.com/index.php/JOBIMB

Probiotic Properties of *Lactobacillus* Isolates from Chicken Intestines

Wan Nur Fadhilah Shamsudin[1,2]*, Loo Shu San[2], Ho Yin Wan[1], Norhani Abdullah[1], Wan Zuhainis Saad[3], Wan Kiew Lian[4]

[1]Institute of Bioscience, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia.
[2]Agro-Biotechnology Institute Malaysia (ABI), c/o MARDI Headquarters,
43400 Serdang, Selangor, Malaysia.
[3]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia.
[4]School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia,
43600 UKM Bangi, Selangor, Malaysia.

*Corresponding author:
Wan Nur Fadhilah Binti Shamsudin
Institute of Bioscience,
Universiti Putra Malaysia,
43400 Serdang, Selangor.
Telephone numbers: +603 - 9769 3045
Facsimile: +603 - 8947 2101
Emel: wnfadhilah@gmail.com

HISTORY

Received: 25th August 2019
Received in revised form: 14th of October 2019
Accepted: 18th of November 2019

KEYWORDS

Lactobacillus
probiotic properties
antagonistic effects
pathogens

ABSTRACT

Three lactic acid bacteria (LAB) isolates which demonstrated probiotic properties were obtained from chicken intestine. The isolates demonstrated good tolerance to acidic pH 3, 0.3% bile salts and strong adhesion to Caco-2 cells. These isolates also showed antagonistic activity against pathogenic *E. coli* (078:K80), *Salmonella enterica serovar Typhimurium*, *C. perfringens* (Type A), *Salmonella enterica serovar Enteritidis*, *E. faecium* (FM3), *E. faecalis* (E227). All three isolates had high inhibition zone (> 6 mm) against *E. coli* 078:K80 and *Salmonella enterica s*erovar Typhimurium and were susceptible to antibiotics gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol and ampicillin. These isolates were identified by using 16S rRNA sequencing technique and as two *L. salivarius* and one *L. reuteri* with accession number MH375403, MH375402 and MH375404, respectively.

INTRODUCTION

The ban or restriction on sub-therapeutic antibiotic usage for poultry production has been imposed in many countries. Environmentally friendly supplements, such as probiotics, have been considered as a possible alternative to antibiotics [1]. Probiotics are live organisms which when administered in adequate amounts confer a health benefit on the host [2]. It has been reported that probiotics are able to improve the health of the host animal by enhancing the beneficial microflora, improving the host resistance to pathogenic microorganisms, and increasing the level of immunomodulation of the host [3]. The most commonly used microorganisms as probiotic are lactic acid bacteria consisting of the genus *Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus.*

In the selection of a potential probiotic strain, the primary criteria are the ability to survive in the gastrointestinal tract (GIT) where acid and biles are present, and the ability to adhere to the intestinal wall. Furthermore, it is important that probiotic candidates are able to impose some beneficial bioactivities such as antimicrobial activity against pathogens and enhanced digestive functions.

The importance of probiotics is well recognized and continuous efforts are pursued to discover effective strains that are capable of rendering specific functions such as combating disease like coccidiosis in poultry. Thus, the present study was carried out to isolate *Lactobacillus* spp. from chicken intestines and to evaluate their probiotic properties.

MATERIALS AND METHODS

Isolation and characterisation of *Lactobacillus* isolates

Lactobacillus isolates were obtained from the chicken intestines according to the method described by Ouled-Haddar et al. [4]. Samples from chicken intestines were collected from five healthy chickens, at 42 days of age, at the local market immediately after being euthanized. The whole intestines of the chickens were collected and kept individually in a sealed plastic bag placed in ice. The intestinal contents of each chicken were scrapped, weighed for 1.0g, and vortexed with 0.9mL of 0.89% NaCl (w/v) for 30 seconds. Then the contents were diluted serially (10^{-1} to

10^{-10}) and 0.1mL of each dilution was spread plated on de Man, Rogosa and Sharpe (MRS) agar medium (Oxoid) agar. The plates were incubated anaerobically in the anaerobic jar with anaerobic gas generating sachets (Oxoid™ AnaeroGen™, Europe) for 24 h at 37ºC incubator. After the incubation period, single colonies were picked, and isolates were purified by two-times purification step on MRS agar and broth. A total of 46 isolates was obtained. The isolates were maintained in 1% (v/v) MRS broth incubated in the anaerobic jar with anaerobic gas generating sachets (Oxoid™ AnaeroGen™, Europe) for 24 h at 37ºC incubator, and stored in 20% glycerol in -80ºC freezer for future use [5].

The isolates were characterised by Gram stain and catalase test according to the methods described as before [6]. Only Gram-positive and catalase negative isolates were used for screening of probiotic properties. Overnight cultures in MRS broth with turbidity of 1.5 at OD_{600nm} were used for each test. *Lactobacillus* isolates were evaluated for their tolerance against Phosphate Buffered Saline (PBS) buffer at pH 1, 2 and 3. The isolates were mixed with the respective buffers for 0, 1, 2 and 3 h before they were cultured in MRS broth overnight [7]. Bile tolerance test and adhesion test (Caco-2 cell line, ATCC HTB-37) were conducted according to the method described by Jin et al. [8] and Duary et al. [9], respectively. Antagonistic effects of isolates against six test pathogens (as listed in **Table 1**) was evaluated according to the method described by El-Kholy et al. [10]. All pathogenic strains were obtained from Laboratory of Vaccines and Immunotherapeutic (LIVES), Institute of Bioscience, University Putra Malaysia (UPM) stock culture. Antibiotic susceptibility test (minimal inhibitory concentration) for the *Lactobacillus* isolates was performed according to the ISO 10932/IDF 223 standard [11]. The minimal inhibitory concentrations (MICs) layout are shown in the **Table 2.**

Table 1. Test pathogens and their specific growth media.

[1]Test pathogens	Origin	Growth medium
Eschericia coli (078:K80)	Chicken	Nutrient broth/agar
Salmonella enterica serovar Typhimurium	Chicken	Nutrient broth/agar
Clostridium perfringens (Type A)	Chicken	Brain heart infusion broth/agar
Salmonella enterica serovar Enteritidis	Chicken	Nutrient broth/agar
Enterococcus faecium (FM3)	Chicken	Brain heart infusion broth/agar
Enterococcus faecalis (E227)	Chicken	Brain heart infusion broth/agar

[1]Laboratory of Vaccines and Immunotherapeutic (LIVES), Institute of Bioscience, Universiti Putra Malaysia (UPM) stock culture.

Table 2. Layout of the microdilution plate with each concentrations of antibiotics used.

Row/	1	2	3	4	5	6	7	8	9	10	11	12
Antibiotic	Concentration of the antibiotics (µg/mL)											
Gentamicin	P	1	2	4	8	16	32	64	128	256	512	N
Kanamycin	P	4	8	16	32	64	128	256	512	1024	2048	N
Streptomycin	P	1	2	4	8	16	32	64	128	256	512	N
Tetracycline	P	0.25	0.5	1	2	4	8	16	32	64	128	N
Erythromycin	P	0.032	0.063	0.125	0.25	0.5	1	2	4	8	16	N
Clindamycin	P	0.063	0.125	0.25	0.5	1	2	4	8	16	32	N
Chloram-phenicol	P	0.25	0.5	1	2	4	8	16	32	64	128	N
Ampicillin	P	0.063	0.125	0.25	0.5	1	2	4	8	16	32	N

Note:
Value in each row represents the concentrations of each antibiotics respectively; P=Positive control in each well without antibiotic and test strain with medium contain solvent that dissolved antibiotic at the highest concentration (Strain + LSM broth only); N=Negative control in each well without test strain or antibiotic (LSM broth only).

Identification of Lactobacillus isolates

Extraction of total genomic DNA

Bacterial cells were harvested from overnight culture (1.5mL) of each isolate in MRS broth by centrifugation at 500 x g for 10 min at room temperature. The cell pellet was used for extraction of total genomic DNA by using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocal. Bacterial cell pellet was pre-treated with lysozyme (20mg/mL) for 30 min. lysis buffer was then added. Proteaniase K was used for digestion of proteins. Total genomic DNA was purified using a mini spin column (Qiagen, Germany), and eluted with elution buffer in a final volume of 100µL. DNA concentration and purity were measured using NanoDrop BioSpectrophotometer (Eppendort, Germany). The extracted DNAs were then kept at -20 ºC for amplification.

Amplification of 16S rRNA gene

For amplification of the 16S rRNA gene, universal primers pA (5' – AGA GTT TGA TCC TGG CTC AG-3') and pE (5'-CCGTCA ATT CCT TTG AGT TT-3'), were used with expected PCR product size of 1.5 kb. The PCR amplification was performed in 25 µL reaction mixtures using a MyCycler Thermal Cycler (BioRad, USA). The PCR reaction mixture contained 2.5 µL reaction mixture of PCR buffer [10X PCR amplification buffer containing 500mM KCl, 100mM Tris-HCl. (pH9.0), and 1% Triton-X-100], 0.5 µL of deoxynucleotide triphosphate (dNTP, i-DNA biotechnology Pte, Ltd, Singapore) in concentration 0f 10mM, 0.5µL of Taq DNA polymerase (Viogene, Taipei, Taiwan, 2U/µL) and 0.5µL of template DNA (corresponding to approximately 50 to 100ng of DNA). The PCR conditions were as follows: initial denaturation at 94ºC for 3 minutes; 29 cycles of denaturation at 45ºC for 45 seconds each; primer annealing at 53ºC for 60 seconds, and primer extension at 72ºC for 90 seconds; and final step of primer extension at 72ºC for 5 minutes before being held at 4ºC.

Sequencing alignments and phylogenetic inference

DNA sequence data sets were assembled using Bioedit sequence alignment editor software. Discrepancy nucleotides between forward and reverse sequence were edited based on their electropherograms. Similarity values were determined using Basic Local Alignment Tool (BLAST) of the GenBank (NCBI). Sequence with $\geq$97% to the previously published sequence were used as the criterion to indicate species identity [12].

A phylogenetic tree was constructed based on the 16S rRNA gene sequence analysis where the analysis involved 17 nucleotide sequences comprising 3 sequences of *Lactobacillus* isolates obtained in this study, 14 sequences belonging to *Lactobacilli* species that were obtained from the GenBank. *Lactococcus lactis* subsp. *lactis* AB100803.1 as outgroup. The 14 *Lactobacilli* reference sequences from the GenBank were under the following accession numbers: *L. salivarius* FJ751785.1, *Lactobacillus* sp. EU600923.1, *L. salivarius* FJ751787.1, *L. salivarius* FJ751784.1, *L. salivarius* FJ751780.1, *L. salivarius* AB612967.1, *L. salivarius* FJ751782.1, *L. salivarius* FJ751779.1, *L. reuteri* KC348394.1, *L. reuteri* EU626021.1, *L. reuteri* EU626015.1, *L. reuteri* JF745115.1, *L. reuteri* EU626022.1, and *L. reuteri* EU626020.1.

RESULTS

A total of 46 isolates that showed typical characteristics of *Lactobacillus* bacterial colonies (circular or slightly irregular shape, flat, convex, or umbonate surface, white or creamy-white in colour), obtained from all five contents of chicken intestine were picked, cultured and purified.

Acid tolerance

Table 3 shows the OD_{600} of the 15 isolates that had been cultured in MRS broth overnight. The 0 h (control) sample was taken immediately after mixing with the buffer. After 1 and 2 h mixing, at pH 1, all isolates showed negligible, growth, except for isolate C1 3.1.4 compared to the control (0 h).

After 3 h of mixing, all isolates did not show any growth. These results indicated that all isolates were susceptible to pH 1, except for isolates C1 3.1.4 that could withstand pH 1 for 2 h of incubation. Isolate CI 3.2.1 showed the highest growth, while isolate CI 4.2.1 had the lowest growth at 0 h.

Table 3. Growth of *Lactobacillus* isolates after mixing with PBS buffer pH 1 for 0, 1, 2 and 3 h.

Isolates	OD_{600} reading			
	pH 1.0			
	Control	1 h	2 h	3 h
CI 1.1.2	1.60±0.01^{a}	0.01±0.00^{b}	0.02±0.00^{b}	0.05±0.01^{b}
CI 1.2.1	1.26±0.00^{a}	0.00±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{b}
CI 1.3.1	1.68±0.02^{a}	0.01±0.01^{b}	0.01±0.00^{b}	0.01±0.01^{b}
CI 1.3.2	1.44±0.08^{a}	0.04±0.00^{b}	0.02±0.01^{b}	0.02±0.00^{b}
CI 2.1.3	1.61±0.00^{a}	0.01±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
CI 2.2.3	1.66±0.02^{a}	0.02±0.00^{b}	0.01±0.01^{b}	0.00±0.00^{b}
CI 3.1.1	1.71±0.01^{a}	0.02±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
CI 3.1.4	1.77±0.00^{a}	1.79±0.00^{a}	1.78±0.00^{a}	0.02±0.00^{b}
CI 3.1.5	1.74±0.01^{a}	0.03±0.00^{b}	0.04±0.00^{b}	0.02±0.00^{b}
CI 3.2.1	1.83±0.01^{a}	0.01±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}
CI 3.3.1	1.35±0.01^{a}	0.02±0.00^{b}	0.03±0.01^{b}	0.02±0.01^{b}
CI 4.1.2	1.68±0.00^{a}	0.32±0.02^{b}	0.00±0.01^{c}	0.01±0.01^{c}
CI 4.2.1	1.05±0.01^{a}	0.01±0.01^{b}	0.01±0.00^{b}	0.00±0.00^{b}
CI 5.1.1	1.79±0.01^{a}	0.00±0.01^{b}	0.00±0.00^{b}	0.00±0.01^{b}
CI 5.3.1	1.58±0.03^{a}	0.00±0.00^{b}	0.00±0.01^{b}	0.01±0.01^{b}

Note:
Values are means±standard error (n=3) Means within a column with different superscripts letters are significantly different (P<0.05). Isolates were mixed with PBS buffer pH 1 for 0 (control), 1,2 and 3 h before culturing in MRS broth overnight. Control samples were taken immediately after mixing with the buffer.

Table 4 shows the OD_{600} of the 15 isolates that had been cultured in MRS broth overnight after mixing with PBS buffer pH 2.0, for 0. 1, 2 and 3 h. The 0 h (control) sample was taken immediately after mixing with the buffer. The results showed that many isolates (CI 1.3.1, CI 1.1.2, CI 1.3.2, CI 3.1.4 and CI 3.1.5) could survive in pH 2 PBS buffer for up to 3 h. Isolates number CI 3.2.1 showed the highest OD reading at 0 h, whereas isolate CI 4.2.1 showed the lowest reading. Other bacterial isolates could not survive after 1 to 3 h of mixing with the buffer.

Table 4. Growth of *Lactobacillus* isolates after mixing with PBS buffer pH 2 for 0, 1, 2 and 3 h.

Isolates	OD_{600} reading			
	pH 2.0			
	Control	1 h	2 h	3 h
CI 1.1.2	1.81±0.01^{a}	1.83±0.01^{a}	1.71±0.01^{b}	1.60±0.01^{c}
CI 1.2.1	1.40±0.01^{a}	1.31±0.00^{b}	0.02±0.01^{c}	0.01±0.00^{c}
CI 1.3.1	1.72±0.00^{a}	1.75±0.00^{a}	1.76±0.01^{a}	1.70±0.00^{a}
CI 1.3.2	1.81±0.01^{a}	1.83±0.01^{a}	1.71±0.01^{b}	1.60±0.01^{c}
CI 2.1.3	1.65±0.01^{a}	1.65±0.01^{a}	1.53±0.01^{b}	1.51±0.03^{b}
CI 2.2.3	1.64±0.01^{a}	0.00±0.00^{b}	0.01±0.00^{b}	0.01±0.01^{b}
CI 3.1.1	1.78±0.01^{a}	1.01±0.01^{b}	0.02±0.01^{c}	0.00±0.01^{c}
CI 3.1.4	1.77±0.01^{a}	1.82±0.01^{a}	1.73±0.01^{a}	1.67±0.00^{b}
CI 3.1.5	1.74±0.00^{a}	1.75±0.00^{a}	1.74±0.00^{a}	1.68±0.01^{b}
CI 3.2.1	1.88±0.01^{a}	1.84±0.01^{a}	1.84±0.00^{a}	0.00±0.01^{b}
CI 3.3.1	1.58±0.00^{a}	1.38±0.01^{b}	1.33±0.01^{b}	1.31±0.01^{b}
CI 4.1.2	1.71±0.01^{a}	1.69±0.00^{a}	1.43±0.02^{b}	1.40±0.01^{b}
CI 4.2.1	1.28±0.01^{a}	0.01±0.01^{b}	0.01±0.01^{b}	0.01±0.01^{b}
CI 5.1.1	1.81±0.01^{a}	1.63±0.01^{b}	0.01±0.00^{c}	0.02±0.01^{c}
CI 5.3.1	1.84±0.00^{a}	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{b}

Note:
Values are means±standard error (n=3) Means within a column with different superscripts letters are significantly different (P<0.05). Isolates were mixed with PBS buffer pH 2 for 0 (control), 1,2 and 3 h before culturing in MRS broth overnight. Control samples were taken immediately after mixing with the buffer.

Table 5 shows the OD_{600} of the 15 isolates that have been cultured in MRS broth overnight after mixing with pH 3.0 PBS buffer, for 0 h (control), 1 h, 2h and 3 h. The control sample was taken immediately after mixing with pH 3.0 PBS buffer. Most isolates could withstand PBS buffer pH 3.0. Isolates number CI 1.1.2, CI 1.3.1, CI 2.1.3, CI 3.1.1, CI 3.1.5, CI 3.2.1 and CI 5.1.1 showed high survival rate after 3 h of mixing with PBS buffer pH 3.0. Other isolates showed varying ability to withstand pH 3 at different mixing periods. Among the isolates, isolate CI 1.1.2, CI 1.3.2, CI 3.1.1. CI 3.2.1 CI 5.1.1 and CI 5.3.1 showed OD reading of >1.80 at 0 h. The lowest OD reading was shown by isolate CI 4.2.1.

Table 5. Growth of *Lactobacillus* isolates after mixing with PBS buffer pH 3 for 0, 1, 2 and 3 h.

Isolates	OD_{600} reading			
	pH 3.0			
	Control	1 h	2 h	3 h
CI 1.1.2	1.88±0.01^{a}	1.85±0.01^{a}	1.86±0.02^{a}	1.84±0.01^{a}
CI 1.2.1	1.44±0.00^{a}	1.35±0.00^{b}	1.32±0.01^{b}	1.26±0.00^{c}
CI 1.3.1	1.72±0.00^{b}	1.76±0.01^{a}	1.75±0.01^{a}	1.75±0.01^{a}
CI 1.3.2	1.85±0.01^{a}	1.82±0.00^{a}	1.83±0.00^{a}	1.71±0.00^{b}
CI 2.1.3	1.68±0.00^{a}	1.66±0.00^{a}	1.71±0.01^{a}	1.69±0.01^{a}
CI 2.2.3	1.65±0.01^{a}	1.64±0.02^{a}	1.62±0.01^{a}	1.58±0.01^{b}
CI 3.1.1	1.88±0.01^{a}	1.85±0.01^{a}	1.86±0.02^{a}	1.84±0.01^{a}
CI 3.1.4	1.79±0.01^{a}	1.63±0.01^{b}	1.72±0.01^{b}	1.78±0.02^{a}
CI 3.1.5	1.74±0.01^{a}	1.77±0.01^{a}	1.72±0.01^{a}	1.75±0.03^{a}
CI 3.2.1	1.89±0.00^{a}	1.86±0.01^{a}	1.85±0.00^{a}	1.86±0.00^{a}
CI 3.3.1	1.46±0.00^{a}	1.41±0.00^{a}	1.29±0.00^{b}	1.27±0.01^{b}
CI 4.1.2	1.75±0.01^{a}	1.75±0.01^{a}	1.42±0.01^{b}	1.40±0.00^{b}
CI 4.2.1	1.27±0.01^{a}	1.28±0.01^{a}	1.16±0.04^{b}	1.04±0.01^{c}
CI 5.1.1	1.81±0.01^{a}	1.82±0.01^{a}	1.84±0.02^{a}	1.84±0.00^{a}
CI 5.3.1	1.85±0.01^{a}	1.82±0.00^{a}	1.83±0.00^{a}	1.71±0.00^{b}

Note:
Values are means±standard error (n=3). Means within a column with different superscripts letters are significantly different (P<0.05). Isolates were mixed with PBS buffer pH 3 for 0 (control), 1,2 and 3 h before culturing in MRS broth overnight. Control samples were taken immediately after mixing with the buffer.

Bile Tolerance

Table 6 shows the OD_{600} of the 15 isolates that have been cultured in MRS broth overnight after mixing with MRS broth with or without bile salt for 1 h, 2 h 3 h and 4 h. The results showed that all isolates could withstand the presence of bile salts after 4 h of mixing. Isolate CI 3.2.1 showed the highest OD reading after 4 h of mixing with bile salt. Isolate CI 3.3.1 showed the lowest growth.

Table 6. Growth of *Lactobacillus* isolates after mixing with MRS broth containing bile salts for 0, 1, 2 and 3 h.

Isolates	OD_{600} reading				
	Without bile salt	With bile salt			
		1 h	2 h	3 h	4 h
CI 1.1.2	1.79±0.00^{a}	1.76±0.02^{a}	1.78±0.01^{a}	1.79±0.01^{a}	1.80±0.01^{a}
CI 1.2.1	1.73±0.03^{a}	1.77±0.00^{a}	1.77±0.00^{a}	1.77±0.00^{a}	1.78±0.00^{a}
CI 1.3.1	1.76±0.01^{a}	1.73±0.02^{a}	1.75±0.01^{a}	1.74±0.02^{a}	1.78±0.01^{a}
CI 1.3.2	1.67±0.01^{b}	1.64±0.00^{b}	1.65±0.00^{b}	1.66±0.00^{b}	1.74±0.01^{a}
CI 2.1.3	1.72±0.01^{a}	1.72±0.00^{a}	1.72±0.01^{a}	1.74±0.02^{a}	1.73±0.00^{a}
CI 2.2.3	1.78±0.01^{a}	1.75±0.01^{a}	1.77±0.01^{a}	1.78±0.01^{a}	1.79±0.01^{a}
CI 3.1.1	1.83±0.04^{a}	1.78±0.05^{b}	1.76±0.03^{b}	1.77±0.03^{b}	1.79±0.02^{b}
CI 3.1.4	1.75±0.00^{a}	1.72±0.00^{a}	1.71±0.00^{a}	1.72±0.00^{a}	1.72±0.00^{a}
CI 3.1.5	1.85±0.05^{a}	1.90±0.03^{a}	1.91±0.05^{a}	1.92±0.03^{a}	1.92±0.03^{a}
CI 3.2.1	1.74±0.01^{a}	1.74±0.01^{a}	1.75±0.00^{a}	1.77±0.01^{a}	1.77±0.00^{a}
CI 3.3.1	1.33±0.02^{a}	1.26±0.02^{b}	1.26±0.05^{b}	1.24±0.11^{b}	1.27±0.12^{b}
CI 4.1.2	1.45±0.13^{c}	1.30±0.01^{d}	1.58±0.05^{b}	1.73±0.02^{a}	1.75±0.02^{a}
CI 4.2.1	1.79±0.01^{a}	1.75±0.00^{a}	1.76±0.01^{a}	1.77±0.01^{a}	1.79±0.01^{a}
CI 5.1.1	1.84±0.01^{a}	1.81±0.01^{a}	1.82±0.00^{a}	1.83±0.00^{a}	1.85±0.01^{a}
CI 5.3.1	1.77±0.00^{a}	1.75±0.02^{a}	1.75±0.01^{a}	1.72±0.02^{a}	1.70±0.01^{a}

Note:
Values are means±standard error (n=3). Means within a column with different superscripts letters are significantly different (P<0.05). Isolates were mixed with MRS broth containing bile salts for 1, 2, 3 and 4 h before culturing in MRS broth overnight.

Adherence assay

The isolates showed varying ability to adhere to the Caco-2 cells. As shown in **Table 7**, isolate CI 3.1.4 showed the best ability to adhere with 25.4±0.13 bacteria attached per cell, followed by isolate CI 2.1.3. with a score of 20.3±0.47 per cell. The lowest score was observed for isolate CI 3.1.5 with a score of 4.45±0.43 per cell. *Lactobacillus fermentum* HM3 (from human milk) showed significantly (P<0.05) lower adhesion score compared to other isolates from chicken intestine. Only the best 3 isolates were selected for further study.

Table 7. Adhesion of cells of *Lactobacillus* isolates to Caco-2 cell.

Isolates	Isolation site	[1]Adhesion score (*Lactobacillus* cells per Caco-2 cell)
CI 1.1.2	Chicken intestine	9.75±0.45[d]
CI 1.3.1	Chicken intestine	12.3±0.46[c]
CI 2.1.3	Chicken intestine	20.3±0.47[b]
CI 3.1.4	Chicken intestine	25.4±0.13[a]
CI 3.1.5	Chicken intestine	4.45±0.43[e]
L. fermentum HM3	Human milk	5.45±0.38[e]

[1]Values are means±standard error (n=3) of adhesion scores. Means within a column with different superscripts letters are significantly different (P<0.05).

Antagonistic effects

The results of antagonistic effects of the 3 isolates against 6 pathogenic strains are shown in **Table 8**. All 3 isolates and reference strain (*L. casei* Shirota) showed antagonistic effects against all pathogens strain tested with variable inhibition zones. *L. casei* Shirota strain showed significantly higher (P<0.05) inhibition zone against *E. coli*, *C. perfringens*, and *E. faecalis* (E227) compared to all other isolates. Isolate CI 1.3.1 showed larger inhibition zones against *Salmonella enterica* serovar *Typhimurium*, *E. faecium* (FM3) and *E. faecalis* (E227) compared to the other isolates. All isolates showed high inhibition (value > 6mm) against *E. coli* 078:K80 and *S. typhimurium*.

Antibiotic susceptibility test

Table 9 shows the susceptibility of minimal inhibitory concentrations (MIC) for all 3 *Lactobacillus* isolates against eight antibiotics. All 3 *Lactobacillus* isolates including the reference strain (*L. casei* Shirota) exhibited MIC values equal or lower than the MIC breakpoints of *Lactobacillus* spp. recommended by the EFSA on all antibiotics; gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol and ampicillin.

Table 8. Antagonistic effects of the isolates against pathogens.

Isolates	Inhibition zone (mm)					
	E. coli 078:K80	*Salmonella enterica* serovar Typhim-urium	*C. perfri-ngens* (Type A)	*Salmonella enterica serovar Enteritidis*	*E. faecium* (FM3)	*E. faecalis* (E227)
CI 1.3.1	6.75±0.25[b]	7.83±0.14	5.08±0.38[b]	6.50±0.44[a]	5.67±0.19[a]	5.75±0.28[b]
CI 2.1.3	6.25±0.25[b]	8.50±0.15	5.17±0.17[b]	5.24±0.13[b]	4.92±0.26[b]	5.17±0.27[bc]
CI 3.1.4	6.17±0.27[b]	8.42±0.19	4.58±0.31[b]	5.25±0.13[b]	4.25±0.13[c]	5.00±0.12[c]
L. casei Shirota	7.83±0.17[a]	8.50±0.15	8.50±0.15[a]	6.17±0.21[a]	5.08±0.08[b]	7.25±0.13[a]

Note:
Values are means ± standard error of 3 replicate plates with 3 spots each Means within a column with different superscripts letters are significantly different (P<0.05). Inhibition category: value < 2mm = no inhibition; value < 5mm = low inhibition capacity; value > 6mm = high inhibition capacity (Carasi et al., 2014).

Table 9. Minimal Inhibitory Concentration (MIC) for antibiotic susceptibility of *Lactobacillus* isolates.

Isolate/ Antibiotic (µg/mL)	[1]MIC (mg L^{-1})							
	CI 1.3.1		CI 2.1.3		CI 3.1.4		[2]*L. casei* Shirota	
	[3]Break point	MIC	[3]Break point	MIC	[3]Brea kpoint	MIC	[3]Break point	MIC
Gentamicin	16	<8	16	<4	8	<8	32	<16
Kanamycin	64	<4	64	<32	64	<32	64	<16
Streptomycin	64	<64	64	<8	64	<64	64	<4
Tetracycline	8	<2	8	<4	16	<16	4	<4
Erythromycin	1	<1	1	<1	1	<1	1	<1
Clindamycin	1	<1	1	<1	1	<1	1	<1
Chloram-phenicol	4	<1	4	<2	4	<4	4	<1
Ampicillin	4	<4	4	<1	4	<2	4	<2

Note:
[1]Values in each column are the MIC of each isolates from two experiments with 3 replicates each.
[2]Reference strain
[3]Values in each column represent the breakpoint of each isolates provided by EFSA for facultative heterofermentative *Lactobacillus* strains.

Identification of Lactobacillus isolates using 16S rRNA gene sequencing

The results of comparative 16S rRNA gene analysis (Table 10) showed all the 3 isolates belonged to the genus *Lactobacillus*. Isolates CI 1.3.1 and CI 2.1.3 were 99% similar to the *L. salivarius* while CI 3.1.4 was 99% similar to *L. reuteri*. The 16S rRNA gene sequences of the 3 *Lactobacillus* isolates were deposited in the GenBank database under the accession numbers MH375403, MH375402, and MH375404 for isolates CI 1.3.1, CI 2.1.3 and CI 3.1.4 respectively.

Phylogenetic analysis based on 16S rRNA gene

Fig. 1 shows the phylogenetic tree based on 16S rRNA gene sequence analysis, depicting the phylogenetic relationships among the 3 *Lactobacillus* type strains obtained from the GenBank. Strains CI 3.1.4 was clustered together with *L. reuteri* JF745115.1 (bootstrap value of 100%). Strain CI 1.3.1 and CI 2.1.3 were clustered together with *Lactobacillus* sp. EU600923.1 and *L. salivarius* AB612967.1 with a bootstrap value of 99%.

Table 10. Identification of *Lactobacillus* isolates using 16S rRNA gene sequencing.

Isolates	Accession number	Nearest matched species from GenBank	Similarity (%)
CI 1.3.1	MH375403	*L. salivarius*	99%
CI 2.1.3	MH375402	*L. salivarius*	99%
CI 3.1.4	MH375404	*L. reuteri*	99%

Similarity values were determined using Basic Local Alignment Tool (BLAST) of the GenBank (NCBI). Sequences with ≥ 97% similarity to the previously published sequences were used as the criterion to indicate species identity.

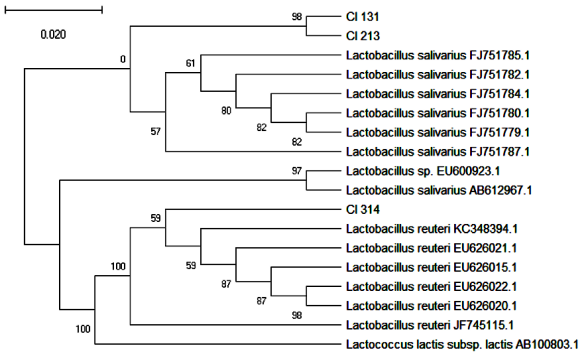

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis depicting the phylogenetic relationships among species of the genus *Lactobacillus* using Neighbor-Joining method [13]. The analysis involved 17 nucleotide sequences including 3 sequences of strain obtained from this study and 14 sequences belong to *Lactobacillus* species obtained from GenBank (NCBI). Bootstrap values above 50% are indicated at the nodes of the tree. *Lactococcus lactis* subsp. *lactis* AB100803.1 as outgroup. The scale bar represents 0.020-nucleotide substitute per position.

DISCUSSION

Most isolates could withstand pH 2 and 3 with varying levels of survival rate depending on the periods of exposure. The tolerance to acidic conditions is important to ensure their survival in the acidic condition of the GIT (pH 1.5 to 2.0). [14] also reported that 20 *Lactobacillus* isolates from chicken and calves showed high survival percentage (48% to 85%) when incubated for 4 h with MRS broth pH 2.5. *Lactobacillus* or any other microorganisms that have been strictly exposed to acidic conditions are more likely to adapt and could withstand acidic stress [15].

Gram-positive bacteria possess multiple acid resistance systems which help them to overcome the stress due to acidic conditions. The most common mechanism includes the usage of proton pumps, the protection of macromolecules, cell membrane changes, production of alkali, induction of pathways by transcriptional regulators, and alteration of metabolism [16]. These mechanisms overcome the negative impact of a reduction in cytoplasmic pH, which can include loss of activity of the relatively acid-sensitive glycolytic enzymes in the energy metabolic pathway and structural damage to the cell membrane and macromolecules such as DNA and proteins [16].

Fifteen isolates treated with 0.3% bile salt could survive for 1 h, 2 h, 3 h, and 4 h of exposure. [14] also reported 20 isolates showed 52% to 91% survival rate after 4 h exposure to 0.3% bile salts. The concentration of bile salts used in these studies was within the physiological concentration of bile salt in the gastrointestinal tract [14]. [17] reported that out of 90 isolates derived from swine intestine, only 16 isolates showed notable survival rate to low pH and bile salt presence up to 6 h incubation. Bacterial response to bile is dependent on a variety of processes addressed towards detoxification of bile and counteracting the deleterious effect on bacterial structures. The mode of action includes active efflux of bile acids or bile salts [18], bile salt hydrolysis by hydrolyses [19], and changes in the architecture or composition of cell membrane and cell wall [20]. In addition, general stress response, protection against oxidative damages, as well as glycolytic reorganizations are other common consequences of bile exposure, that might be employed to counteract some of the cellular damage caused by these compounds.

The ability to tolerate low pH and the presence of bile salts are important criteria to consider in evaluating LAB as probiotics [21]. In the present study, four isolates (CI 3.1.4, CI 2.1.3, CI 1.3.1 and CI 1.1.2) showed higher cells adhesion index than the reference strain *L. fermentum* HM3. A study conducted by [22] showed that the percentage of adhesion of *L. curratus* was 16% compared to 7% by the *L. rhamnosus* GG as control strain. [23] discussed that the adhesion score varied depending on the source of LAB isolates. A commercial reference strain *L. casei* Shirota had adherence ability of 13.1 to 19.7 attached bacteria per cell, while *L. fermentum* which was isolated from human milk had an average of 37.7 per cell. Adhesion to cell is another important criterion in selecting probiotic candidates as it is necessary for the cells to adhere to the intestinal lining for colonization. Probiotic colonization confers protection and microbial balance in the GIT of the host [24]. The adhesion index or score by using Caco-2 cells as an *in vitro* model for the ability to adhere to intestinal cells is known to have a good correlation to *in vivo* results [22].

In the antagonistic effect evaluation, the pathogenic strains chosen are commonly detected as the causal agents in poultry infection. All three isolates and the reference strain (*L. casei* Shirota) exhibited antimicrobial properties towards all pathogenic strains tested. This ability conferred the probiotic characteristic of the LAB. Various reports showed that most *Lactobacilli* possess antimicrobial potential against different foodborne pathogens [25]. However, not all LAB possesses antimicrobial activity as Aka-gbezo et al. [26] observed that only 9 LAB isolates from fermented maize showed inhibitory effect against *E. faecium* and *E. faecalis*, while 188 isolates did not show any antimicrobial properties towards the pathogenic strains. Degree of inhibition may differ among isolates as the antimicrobial metabolites or bacteriocins productions of each isolate differ [27]. The production of antimicrobial metabolites such as organic acids, ethanol, diacetyl, bacteriocins or proteinaceous substances by the LAB differ [27,25]. The antagonistic capability of LAB is an important criterion besides tolerance to the acidic and bile conditions and cell adhesion ability. The probiotic candidates should be able to inhibit the growth of bacteria associated with infections to overcome one of the main loses of the poultry industry [27].

The determinations of minimal inhibitory concentrations (MIC) of three isolates showed that all were susceptible to antibiotics tested. As recommended by EFSA, isolates susceptible to all antibiotics will not be subjected to any other test for safety evaluation. These findings are in agreements with that of [23] who reported that all 9 *Lactobacillus* isolates and reference strain exhibited MIC value lower than that recommended by the EFSA. In contrast to these findings, [28] recorded that 6 of *Lactobacillus* isolates tested were considered resistance to a particular antibiotic when MIC (µg/mL) values obtained were higher than the recommended breakpoint value. *Lactobacillus plantarum* CE84, *L. curvatus* CE83, *L. brevis* CE94, CE85 were resistant to clindamycin. Clindamycin is usually used as antibiotics for anaerobic bacteria, or even Gram-positive cocci such as *Staphylococcus* or *Streptococcus*. Three *Lactobacillus* isolates obtained from the chicken intestines were identified using 16S rRNA sequencing technique. Isolates CI 1.3.1 and CI 2.1.3 were identified as *L. salivarius* and isolate CI 3.1.4 as *L. reuteri*. Identification of bacterial species by using 16S rRNA gene sequencing confirm their specific phylogenies [29]. [30] isolated 13 LAB that possessed the characteristics consistent with *Lactobacillus* genus and identification was confirmed by means of genomic DNA extraction and PCR analysis. The isolates were identified as *L. reuteri*, *L. vaginalis*, *L. johnsonii*,

and *L. fermentum*. In bacterial identification, morphological and biochemical characteristics are not conclusive and molecular techniques should be applied to confirm the species.

CONCLUSION

Fifteen out of 46 isolates showed good growth rate at 24 h incubation with OD_{600nm} reading above 1.5. The isolates showed the ability to survive acidic condition of pH 2 and 3 (after 3 h exposure) and the presence of 0.3% bile salts (after 4 h exposure). Four isolates showed higher adhesion score of more than 10 cells per Caco-2 cell. Three isolates selected showed antagonistic activity against pathogenic strains. Identification of these isolates by using 16S rRNA gene sequencing technique, showed 99% similarity of CI 1.3.1 as *L. salivarius*, CI 2.1.3 as *L. salivarius* and CI 3.1.4 as *L. reuteri* with accession number MH375403, MH375402, and MH375404, respectively.

ACKNOWLEDGEMENT

We are thankful to the Ministry of Science, Technology and Innovation of Malaysia for the financial support under the ScienceFund grant scheme under the project "Evaluation of probiotic formulation for the control of coccidiosis in broiler chickens" (02-05-19-SF007).

REFERENCES

1. Tierney, J., Gowing, H., Van Sinderen, D., Flynn, S., Stanley, L., McHardy, N., and Mulcahy, G. In vitro inhibition of *Eimeria tenella* invasion by indigenous chicken *Lactobacillus* species. Vet Parasitol. 2004. 122(3): 171–182.
2. FAO/WHO (Food and Agriculture Organization/World Health Organization). Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria.Report of a Joint FAO/WHO expert consultation on evaluation of healt and nutritional properties of probiotics in food including powder milk with lactic acid bacteria. 2001. 1-34.
3. Markowiak, P., and Śliżewska, K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog. 2018. 10(1): 21.
4. Ouled-Haddar, H., Idoui, T., Sifour, M., Guezira, M., and Bouthabet, M. Isolation, characterization and microencapsulation of probiotic *Lactobacillus curvatus* G7 from chicken crop. Online J Sci Technol. 2012. 2(1)1-6.
5. Heravi, R.,M., Kermanshahi, H., Sankian,M., Nassiri,M.,R., Moussavi.,A.,H., Nsiraii,L.,R., Varasteh.,A.,R. Screening of lactobacilli bacteria isolated from gastrointestinal tract of broiler chickens for their use as probiotic. Afr J Microbiol Res. 2011. 5(14): 1858-1868.
6. Black, J.G. Microbiol. John Wiley and Sons (Asia) Pte. Ltd. Seventh Edition. 2008.
7. Ehrmann, M. a., Kurzak, P., Bauer, J., and Vogel, R. F. Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J Appl Microbiol. 2002. 92(5): 966–975.
8. Jin, L. Z., Ho, Y. W., Abdullah, N., and Jalaludin, S. Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. Lett Appl Microbiol. 1998. 27(3): 183–185.
9. Duary, R. K., Rajput, Y. S., Batish, V. K., and Grover, S. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. Indian J Med Res. 2011.134(11): 664–671.
10. El-Kholy, A. M., El-Shinawy, S. H., Meshref, A. M. S. M. S., and Korny, A. M. M. Screening of antagonistic activity of probiotic bacteria against some food-borne pathogens. J Appl and Environmental Microbiol. 2014. 2(2): 53–60.
11. Carasi, P., Díaz, M., Racedo, S. M., Antoni, G. De, Urdaci, M. C., and Serradell, M. de los A. Safety characterization and antimicrobial properties of kefir-isolated *Lactobacillus kefiri*. BioMed Res Int. 2014: 1–7.
12. Abbas Hilmi, H. T., Surakka, A., Apajalahti, J., and Saris, P. E. J. Identification of the most abundant Lactobacillus species in the crop of 1- and 5-week-old broiler chickens. Appl Environ Microbiol. 2007. 73(24): 7867–7873.
13. Saitou, N., and Nei, S. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987.4(4):406-425.
14. Bujnakova, D., Strakova, E., and Kmet, V. *In vitro* evaluation of the safety and probiotic properties of Lactobacilli isolated from chicken and calves. Anaerobe. 2014. 29: 118–127.
15. Sanhueza, E., Paredes-osses, E., González, C. L., and García, A. Electronic J bioTechnol effect of pH in the survival of *Lactobacillus salivarius* strain UCO _ 979C wild type and the pH acid acclimated variant. Electron J BioTechnol. 2015. 18(5): 343–346.
16. Cotter, P. D., and Hill, C. Antimicrobial activity - The most important property of probiotic and starter lactic acid bacteria. Food Technol BioTechnol. 2003. 67(3): 429–453.
17. Wang, Z., Wang, L., Chen, Z., Ma, X., Yang, X., Zhang, J., and Jiang, Z. *In vitro* evaluation of swine-derived *Lactobacillus reuteri*: Probiotic properties and effects on intestinal porcine epithelial cells challenged with enterotoxigenic *Escherichia coli* K88. J Microbiol BioTechnol. 2016. 26(6): 1018–1025.
18. Pfeiler, E. A., and Klaenhammer, T. R. Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. Appl Environ Microbiol. 2009. 75(18): 6013–6016.
19. Lambert, J. M., Bongers, R. S., De Vos, W. M., and Kleerebezem, M. Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. Appl Environ Microbiol. 2008. 74(15): 4719–4726.
20. Ruiz, L., Margolles, A., and Sánchez, B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Front Microbiol 4 (2013): 396
21. Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., And, S. M.-Q., and Gil, A. Sources , isolation , characterisation and evaluation of probiotics. Br J Nutr. 2013. 109: S35–S50.
22. Zommiti, M., Connil, N., Hamida, J. Ben, and Ferchichi, M. Probiotic Characteristics of *Lactobacillus curvatus* DN317, a Strain Isolated from Chicken Ceca. Probiotics Antimicrob Protein. 2017. 9(4): 415–424.
23. Shokryazdan, P., Sieo, C. C., Kalavathy, R., Liang, J. B., Alitheen, N. B., Faseleh Jahromi, M., and Ho, Y. W. Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. BioMed Res International. 2014: 1-16.
24. Thakur, N., Angad, G., Veterinary, D., Rokana, N., Panwar, H., Angad, G., and Veterinary, D. Probiotics: Selection criteria, safety and role in health and disease, J Innovative Biol. 2016. 3(1): 259-270.
25. Andrabi, S. T., Bhat, B., Gupta, M., and Bajaj, B. K. Phytase-producing potential and other functional attributes of lactic acid bacteria isolates for prospective probiotic applications. Probiotics Antimicrob Protein. 2016. 8(3): 121–129.
26. Aka-gbezo, S., Konan, A. G., Cho, M. N., Achi, P., Koffi-nevry, R., Koussemon-camara, M., and Bonfoh, B. Screening of antimicrobial activity of lactic acid bacteria isolated from anango baca slurry, a spontaneously fermented maize product used in Côte d'Ivoire. Int J Biol Chem Sci. 2017. 11(6): 2616–2629.
27. Dec, M., Puchalski, A., Urban-Chmiel, R., and Wernicki, A. Screening of *Lactobacillus* strains of domestic goose origin against bacterial poultry pathogens for use as probiotics. Poultry Sci. 2014. 93(10): 2464–2472.
28. Manini, F, M.,C., Casiraghi, K., Poutanen, M., Brasca, D., Erba, C., Plumed-Ferrer. Characterization of lactic acid bacteria isolated from wheat bran sourdough. Food Sci Technol. 2015. 66: 275 – 283.
29. Chandok, H., Shah, P., Akare, U. R., Hindala, M., Bhadoriya, S. S., Ravi, G. V., and Nayarisseri, A. Screening, isolation and identification of probiotic producing *Lactobacillus acidophilus* Strains EMBS081 and EMBS082 by 16S rRNA Gene Sequencing. Interdisciplinary Scis: Comp Life Sci. 2014.7(3): 242–248.
30. Rajoka, M. S. R., Hayat, H. F., Sarwar, S., Mehwish, H. M., Ahmad, F., Hussain, N.,Shi, J. Isolation and evaluation of probiotic potential of lactic acid bacteria isolated from poultry intestine. Microbiol (Russian Federation). 2018. 87(1): 116–126.