Malaysian Journal of Microbiology, Vol 12(1) 2016, pp. 15-23

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Characterization of conjugated linoleic acid-producing lactic acid bacteria as potential probiotic for chicken

Yong Su Ting[1], Wan Zuhainis Saad[2,3]*, Sieo Chin Chin,[2,4] and Ho Yin Wan[4]

[1]Strategic Livestock Research Centre, Mardi Headquarters, Persiaran Mardi-UPM, 43400 Serdang, Malaysia.
[2]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[3]Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[4]Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
Email: zuhainis@upm.edu.my

Received 6 October 2014; Received in revised form 29 May 2015; Accepted 29 May 2015

ABSTRACT

Aims: Dietary intake of conjugated linoleic acid (CLA) by human is insufficient to exhibit properties of anti-cancer, anti-inflammatory, anti-atherosclerosis, anti-obesity and enhancing immune system. Thus, enrichment of CLA in chicken by bacteria is a suggestion to solve the problem. It would be an advantage to have bacteria capable of producing CLA and has probiotic potential in chicken. Thus, probiotic properties of CLA-producing bacteria were accessed in this study.
Methodology and results: In this study, 47 lactic acid bacteria (LAB) isolated from gastrointestinal tract of chickens were screened for conjugated linoleic acid (CLA) production. *Lactobacillus salivarius* strain P2, *Enterococcus faecium* strain P1 and *Lactobacillus agilis* strain P3 were shown to produce 21.97, 23.35 and 31.08 µg/mL of CLA in MRS broth containing free linoleic acid (0.5 mg/mL) and 2% (w/v) Tween 80, respectively. *Lactobacillus salivarius* strain P2, *E. faecium* strain P1 and *L. agilis* strain P3 were found to be able to tolerate 0.3% oxgall (Difco, France) and pH 2.5. *Lactobacillus agilis* strain P3 and *L. salivarius* strain P2 showed better acid tolerance compared to *E. faecium* strain P1. Besides that, *L. agilis* strain P3 and *L. salivarius* strain P2 were resistant to two out of eight types of antibiotics tested, able to produce 220.04 mM lactic acid and 200.17 mM of lactic acid, respectively. *Enterococcus faecium* strain P1 was resistant to five out of eight types of antibiotic tested, produced 90.39 mM lactic acid and showed hemolytic activity. Only *L. agilis* strain P3 can produce acetic acid at a concentration of 2.71 mM.
Conclusion, significance and impact of study: These results showed that the CLA-producing *L. salivarius* strain P2 and *L. agilis* strain P3 could be potential probiotic bacteria for chickens, which may eventually lead to production of chicken with better meat quality.

Keywords: conjugated linoleic acid, probiotic, lactic acid bacteria, chicken

INTRODUCTION

Chickens are known to be the most consumed meat in the world. The world chicken consumption for year 2013 was reported to be about 94 million tonnes (http://www.thepoultrysite.com/articles/2929). This trend is predicted to increase year by year. In order to increase the current level of chicken production and to meet the huge demand, it is essential that the production runs in a healthy, steady and sustainable manner. In recent years, consumers prefer high quality and safe-to-eat food products. The production of chickens has slowly shifted towards the application of natural supplement such as probiotics to enhance the growth performance of the birds (Dhama *et al.*, 2011).

Probiotic functions to protect the host from pathogens and diseases, as well as increase their feed efficiency, leading to an improvement in the production of meat and egg (Chaucheyras-Durand and Durand, 2010). The beneficial effects of probiotics have prompted the screening of potential probiotic bacteria from various sources. The main criteria in which these strains should at least fulfil are their tolerance toward low gastrointestinal pH and bile, antibiotic resistant and antibacterial activity (Holzapfel and Schillinger, 2002). To date, many probiotic strains with these characteristics have been reported. Apart from these basic requirements, it would be an advantage if these strains could harbour additional capability such as production of conjugated linoleic acid. Conjugated linoleic acid (CLA) is a group of fatty acid isomers of octadecadienoate (C18:2) with double conjugated bonds in different positional and geometric configurations. CLA has been proven in several cell culture systems and animal models to have potential

*Corresponding author

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

properties in anti-cancer, anti-inflammatory, anti-atherosclerosis, anti-obesity and modulation of immune system (Oh *et al.*, 2003). Currently, CLA is recognized as a nutritional supplement due to its functional properties. CLA are normally found in ruminant meats and dairy products but at lower degree in chickens and eggs (Chin *et al.*, 1992). Since chickens and eggs are the most popular protein sources being consumed worldwide, enrichment of CLA in chicken would definitely be a feasible approach to increase dietary CLA in human. Many studies have been carried out to produce CLA enriched meat and eggs. CLA enriched chicken can be accomplished by manipulation of diet which involves supplementation of linoleic acid, linolenic acid and synthetic CLA (Khanal and Olson, 2004) or introducing CLA-producing bacteria into chicken in diet. Although chemically synthesized CLA are available, chemical synthesis of CLA may produce different isomers of CLA which would exert different cell signaling pathway in human that leads to different effects on cell functions (Wahle *et al.*, 2004). The purity of CLA isomers is crucial for human health to exhibit biological function in human. Researchers believe that *c*9, *t*11 CLA, which is referred to as rumenic acid and *t*10, *c*12 CLA are the most biologically active forms and have been reported to exhibit beneficial effects (Kennedy *et al.*, 2010). In this aspect, biological production by bacteria has been reported to produce desired isomers (Ogawa *et al.*, 2005). Previous studies showed that CLA-producing *Bifidobacterium breve* and *Lactobacillus rhamnosus* could produce high concentration of *cis*-9, *trans*-11 CLA (*c*9, *t*11 CLA) (Wall *et al.*, 2009) and *trans*-10, *cis*-12 CLA (*t*10, *c*12 CLA) (Lee *et al.*, 2006; Lee *et al.*, 2007) in animal tissues, respectively.

By far, to the best of our knowledge, bacteria sourced from animals that have been reported to produce CLA are such as *Lactobacillus* (Pariza and Yang, 1999; Puniya *et al.*, 2008; Romero-Pérez *et al.*, 2013), *Propionibacterium acnes* (Wallace *et al.*, 2007), *Bifidobacterium pseudolongum* (Gorissen *et al.*, 2010) and *Clostridium proteoclasticum* (Wallace *et al.*, 2007), *Megasphaera elsdenii* (Kim *et al.*, 2002), *Butyrivibrio fibrisolvens* (Polan *et al.*,1964; Kepler *et al.*, 1966; Asanuma *et al.*, 2001; Fukuda *et al.*, 2005), *Pseudobutyrivibrio* (Cepeljnik *et al.*, 2003; Koppová *et al.*, 2006) and *Eubacterium lentum* (Eyssen and Verhulst, 1984). *Bifidobacterium pseudolongum* subsp. *Pseudolongum* LMG 11595 was the only CLA-producing bacteria isolated from chicken faeces (Gorissen *et al.*, 2010). Only three studies on CLA-producing lactic acid bacteria from animals were reported and the bacteria were isolated from cow milk (Romero-Pérez *et al.*, 2013), rumen of cattle (Puniya *et al.*, 2008) and intestinal tract of rat (Pariza and Yang, 1999). As it is believed that probiotic strains which were isolated from the intended host would have higher survival rate during application, the present study was initiated to screen for potential CLA-producing probiotic strains from chickens.

MATERIALS AND METHODS

Source of bacteria

A total of 47 lactic acid bacteria isolated from gastrointestinal tract of chickens were obtained from Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia. All the cultures were subcultured three times (1%, v/v, inoculum) in MRS broth (Merck, Germany) prior to use. The cultures were incubated at 37 °C for 1 day in an anaerobic jar with gas packs (Anaerocult A, Merck, Germany) to create an anaerobic condition.

Screening of CLA-producing-bacteria

Screening of CLA-producing LAB was carried out as described by Barret *et al.* (2007). One percent (v/v) of the bacteria was incubated anaerobically in MRS broth containing free linoleic acid (0.5 mg/mL) and 2% (w/v) Tween 80 at 37 °C for 48 h to determine the ability of strains to convert linoleic acid to CLA. Following incubation, extraction of lipid from culture media was performed as described by Rodríguez-Alcalá *et al.* (2011). The upper hexane layers were used for the screening of CLA production by UV spectrophotometer and for CLA isomers determination by gas chromatography (GC). CLA amount in the hexane layer were measured spectrophotometrically at the wavelength of 233 nm. The concentration of CLA was determined from a standard curve that showed the relationship between concentration of CLA (*cis*-9, *trans*-11 CLA) and absorbance value (Rodríguez-Alcalá *et al.*, 2011).

Analysis of CLA by GC

Gas chromatography analysis of CLA was carried out to determine concentration and isomers of CLA produced by CLA-producing bacteria. Before GC analysis, methyl esters of CLA were prepared by the modified method of Zakaria *et al.* (2007). After the lipid was extracted from samples (Rodríguez-Alcalá *et al.*, 2011), 100 µL of 4 mg/mL heneicosanoic acid (C21:0) (Sigma, UK) was added to the hexane layer as internal standard (IS) and the hexane layer was dried at 40 °C under a nitrogen flow. Two milliliters of 0.66 N KOH in methanol was added to the teflon-capped tube and incubated at 70 °C for 10 min and the tube was shaken occasionally. Then, 2 mL 15% boron trifluoride was added to the test tube and incubated at 70 °C for 20 min. Four millilitres of distilled deionized water and 4 mL of petroleum ether were added, mixed for 1 min, and centrifuged for 3000 x *g* for 10 min for phase separation. The top petroleum ether layer containing methylated fatty acids was transferred to a new test tube containing about 0.5 g anhydrous sodium sulphate and kept in GC vial for gas chromatographic analysis.Methyl esters of CLA were analyzed using a GC (6890; Wilmington, DE, USA) equipped with a flame-ionization detector (7673 FID), autosampler (7683), automatic injector (7683), split injection port and a flexible

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

silica capillary column (SP2560; 100 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA) coated with poly(biscyanopropyl siloxane). Helium was used as the carrier gas (2.1 mL/min). Operating conditions of GC were set according to Macouzet *et al.* (2009) except helium was used instead of hydrogen as carrier gas. Peaks were identified according to pure methyl ester CLA standard (O5632, Sigma, UK).

16S rRNA gene identification of CLA-producing bacteria

Conjugated linoleic acid-producing bacteria were incubated in MRS broth at 37 °C for 24 h, and centrifuged at 8500 x *g* for 20 min. Approximately 0.2 g of the bacterial pellet was placed in a 1.5 mL microcentrifuge tube. A DNeasy® Blood and Tissue Kit (Qiagen Gmbh, Hilden, Germany) was used to extract bacterial genomic DNA following the manufacturer's instructions. The extracted DNA was electrophoresed in 0.8% agarose gel and visualised using Alpha Imager 1200 documentation and analysis system (Alpha Innotech, USA).

Polymerase chain reaction (PCR) amplification of 16S rRNA gene was performed using a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Each of the reaction mixtures (20 µL) contained 200 µM dNTPs (Finnzymes, Finland), 0.2 µM (each) forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), 1 U of *DyNAzyme II* DNA polymerase (Finnzymes, Finland), 1 × PCR buffer (10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100), 1 µL of about 200 ng template DNA, and the volume was adjusted with deionized water. Initial denaturation was carried out at 94 °C for 4 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 30 sec, 72 °C for 2 min and final extension at 72 °C for 5 min. Reaction mixtures were subsequently cooled to 4 °C. The PCR products were analyzed by agarose gel electrophoresis with 1% agarose with ethidium bromide staining. Purified PCR products were then cloned using TOPO TA Cloning Kit (Invitrogen Ltd, Carlsbad, CA USA) for sequencing.

The sequenced data were analysed and aligned using alignment editor, BioEdit (Hall, 1999). The sequences were compared with the sequences available in the GenBank (National Center of Biotechnology Information, NIH, Bethesda, Maryland) using the Basic Local Alignment Search Tool (BLAST). All sequences were aligned using CLUSTAR W. The results are presented in a similarity matrix. The 16S rRNA gene sequence similarity values were calculated by pairwise comparison of the sequences within the alignment. Identification was based on the highest percentage of similarity above 97%.

Characterization of CLA-producing bacteria

Characterization of CLA-producing bacteria were assayed in at least triplicate with the following tests.

Acid tolerance test of bacteria

The acid tolerance test was carried out according to the method described by Gaudana *et al.* (2010). The number of viable bacteria was determined at 0 h and 2 h after incubation of cultures in acidic buffer with pH 2.0, pH 2.5 and pH 3.0. The survival rate at each pH was calculated as the percentage of number of LAB colonies grown on MRS agar after 2 h incubation in comparison to initial bacterial number.

Bile tolerance test of bacteria

The method for the determination of bile tolerance was as described by Oh *et al.* (2000). The number of viable bacteria was determined at 0 h, 2 h and 24 h after incubation of cultures in MRS broth containing 0.3% oxgall (Difco, France). The survival rate was calculated as the percentage of number of LAB colonies grown on MRS agar after 2 h and 24 h incubation time compared to the number of LAB colonies at 0 h.

Antibiotic susceptibility test

Antibiotic susceptibility testing was carried out by disk diffusion test (CLSI, 2012). Inoculum suspensions of CLA-producing LAB were swabbed onto Mueller-Hinton agar (Oxoid, USA) supplemented with 10% (v/v) MRS dehydrated broth and adjusted to pH 6.7 (Rodríguez-Alonso *et al.*, 2009). Antibiotic discs (Oxoid, USA) from antibiotic group I (30 µg vancomycin, 10 µg ampicillin and 10 units penicillin G), antibiotic group II (30 µg tetracycline, 10 µg gentamicin and 10 µg streptomycin) and antibiotic group III (30 µg chloramphenicol and 15 µg erythromycin) were placed on Mueller-Hinton agar (Oxoid) supplemented with 10% (v/v) MRS dehydrated broth by disc dispenser (Oxoid, USA). The plates were then incubated anaerobically at 37 °C for 48 h. Antibiotic group I are inhibitors of cell-wall synthesis whereas antibiotic group II are inhibitors of bacterial synthesis on the 30S ribosomal subunit. Antibiotic group III are inhibitors of the bacterial synthesis on the 50S ribosomal subunit. Upon 48 h incubation at 37 °C, a measurement of the diameter of the zone of inhibition in millimeters was made, and its size was compared to Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard-Eleventh Edition (M02-A11) and Twenty-Second Informational Supplement (M100-S22) (CLSI, 2012).

Antibacterial activity test

An overlay method was used to determine the ability of cultures to inhibit the growth of pathogens. The indicator bacteria used in the study were *Klebsiella pneumonia* K36, *Serratia marcescens* S913, *Salmonella Enteriditis* IMR S1003/07, *Salmonella typhimurium* S1000, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* 0157:H7, *Proteus mirabilis* P184, *P. vulgaris* P147, methicillin-resistant *Staphylococcus aureus* (MRSA) IMR

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

S1228/07B, *S. aureus* S244, *S. aureus* IMR S1351, *S. epidermidis* S168, *E. feacalis* E227 and *Listeria monocytogenes* L55. After incubation, the plates were examined for presence of > 2 mm clear zones of inhibition around the cultures streaks.

Lactic acid and acetic acid production

Production of lactic acid and acetic acid by cultures was analyzed by GC. One percent inoculum from an overnight MRS culture was inoculated into MRS broth (pH 6.2) and incubated anaerobically at 37 °C for 24 h. Concentration of lactic acid and acetic acid in the cell free-supernatants were quantified using Agilent 6890 Series Gas Chromatograph fitted with a FID. The pH values of the supernatants were also measured. Conditions of GC used in this study were as described by Jin *et al.* (1996) except that DB-FFAP column (30 m × 0.25 µm × 0.25 µm; Agilent Technologies) was used to analyze CLA isomers and concentration produced by CLA-producing bacteria. All tests were performed in triplicate.

Hemolytic activity of bacteria

Hemolysis was evaluated with blood agar plates prepared from blood agar base (Merck, Germany) and 5% (v/v) sterile defibrinated horse blood. The cultures were streaked on the blood agar plates. *Staphylococcus aureus* S244 was used as positive control. The plates were incubated anaerobically at 37 °C for 48 h.

Statistical analysis

One way analysis of variance (ANOVA) was performed using SAS 9.3 to analyze the results obtained from acid tolerance test, bile tolerance test, lactic acid and acetic acid production. Tukey test at the 5% significance level was applied to experimental data to assess statistically significant differences among results. All tests were performed in triplicate and the values were expressed as the mean ± standard deviation.

RESULTS

Screening and identification of CLA-producing bacteria

A total of 3 out of 47 isolates showed the ability to produce CLA. Based on the GC analysis, three isolates, CCB1, CCL6 and P1 were able to produce 21.97, 31.08 and 23.35 µg/mL of CLA in the reaction mixture, respectively. The *c*9, *t*11 CLA isomer was the major isomer generated by isolate CCB1, CCL6 and P1 with 60.65%, 66.90% and 49.77%, respectively. While the *t*10, *c*12 CLA isomer accounted for 39.35%, 33.10% and 50.23% for isolate CCB1, CCL6 and P1, respectively. Based on 16S rRNA gene sequences, isolate CCB1, CCL6 and P1 were identified as *Lactobacillus salivarius*, *L. agilis* and *Enterococcus faecium*, respectively. The 16S rRNA gene sequences of isolate CCB1, CCL6 and P1 were deposited in NCBI gene bank with accession number JQ837457, JQ837458 and JQ837456, respectively and renamed as *L. salivarius* strain P2, *L. agilis* strain P3 and *E. faecium* strain P1, respectively.

Acid tolerance of bacteria

The acid tolerance of the bacteria at pH 2.0, 2.5 and 3.0 after 2 h is as shown in Table 1. Generally, all selected isolates could not survive at pH 2.0 for 2 h. At pH 2.5, 22.98% and 53.82% of *L. salivarius* strain P2 and *L. agilis* strain P3 were found to survive under this experiment condition. The two isolates also showed high survival rate (83.08-87.95%) at pH 3.0. Only 0.01% and 0.25% of *E. faecium* strain P1 was found to survive for 2 h at pH 2.5 and 3.0, respectively.

Table 1: Bacteria population and survival rate of bacteria strains under acidic conditions.

Strain	Acid tolerance					
	pH 3.0		pH 2.5		pH 2.0	
	Bacteria population (log_{10} CFU/mL)	Survival rate (%)	Bacteria population (log_{10} CFU/mL)	Survival rate (%)	Bacteria population (log_{10} CFU/mL)	Survival rate (%)
	Mean ± SD		Mean ± SD		Mean ± SD	
E. faecium strain P1	4.02 ± 0.07	0.25[b]	2.88 ± 0.27	0.01[c]	ND	ND
L. salivarius strain P2	6.73 ± 0.13	83.08[a]	6.11 ± 0.1	22.98[b]	ND	ND
L. agilis strain P3	6.69 ± 0.02	87.95[a]	6.69 ± 0.06	53.82[a]	1.85 ± 0.39	1.29×10^{-3}[a]

ND, non-detectable. n = 9

[a,b,c]Mean with different superscripts within a column are significantly different (P<0.05)

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Bile tolerance of bacteria

The isolates showed different ability to tolerate bile (Table 2). Among the three isolates, *L. salivarius* strain P2 and *L. agilis* strain P3 showed weaker tolerance in which only 45.12-46.38% and 0.52-7.59% of cells survived after 2 h and 24 h incubation, respectively. *E. faecium* strain P1 exhibited better tolerance with 92.13% and 186.61% survival rate at 2 h and 24 h, respectively.

Table 2: Bacteria population and survival rate of bacteria strains in the presence of 0.3% oxgall.

Strain	Bile tolerance 0.3% (w/v)					
	0 h		2 h		24 h	
	Bacteria population (log_{10} CFU /mL)	Survival rate (%)	Bacteria population (log_{10} CFU /mL)	Survival rate (%)	Bacteria population (log_{10} CFU /mL)	Survival rate (%)
	Mean ± SD		Mean ± SD		Mean ± SD	
E. faecium strain P1	6.93 ± 0.03	100.00	6.89 ± 0.04	92.13[a]	7.20 ± 0.02	186.61[a]
L. salivarius strain P2	7.00 ± 0.03	100.00	6.64 ± 0.10	45.12[b]	5.88 ± 0.25	7.59[b]
L. agilis strain P3	7.00 ± 0.12	100.00	6.67 ± 0.08	46.38[b]	4.71 ± 0.11	0.52[c]

ND, non-detectable. n = 9.
[a,b,c]Mean with different superscripts within a column are significantly different (P<0.05).

Antibiotic susceptibility test of bacteria

The results obtained for antibiotic susceptibility test of bacteria are presented in Table 3. Of the 8 antibiotics tested, *E. faecium* strain P1 was resistant to all except vancomycin and chloramphenicol (susceptible) and erythromycin (intermediate reaction). *Lactobacillus salivarius* strain P2 showed resistance to vancomycin and streptomycin, intermediate reaction to erythromycin and susceptible to the others. *Lactobacillus agilis* strain P3 was susceptible to all the tested antibiotics except vancomycin and streptomycin (resistant).

Antibacterial activity of bacteria

The isolates showed a broad antibacterial action in which all the isolates produced inhibition zones of >2 mm against all the tested indicator bacteria (Data not shown).

The antibacterial action of the isolates was found not to be contributed by bacteriocin as this compound was not detected in the isolates. However, the isolates, especially *L. salivarius* strain P2 and *L. agilis* strain P3 produced high amount of lactic acid (200.17 mM and 220.04 mM, respectively). *Enterococcus faecium* strain P1 produced only 90.39 mM of lactic acid. Low concentration of acetic acid, 2.71 mM was detected in *L. agilis* strain P3 (Table 4).

Hemolytic activity of bacteria

Lactobacillus salivarius strain P2 and *L. agilis* strain P3 were tested negative for hemolytic test but *E. faecium* strain P1 displayed α-hemolysis on horse blood agar under anaerobic condition after 48 h incubation at 37 °C.

Table 3: Antibiotic susceptibility of cultures.

Antibiotic	Strain		
	E. faecium strain P1	*L. salivarius* strain P2	*L. agilis* strain P3
Antibiotic group I			
Vancomycin	S	R	R
Ampicillin	R	S	S
Penicillin G	R	S	S
Antibiotic group II			
Tetracycline	R	S	S
Gentamicin	R	S	S
Streptomycin	R	R	R
Antibiotic group III			
Chloramphenicol	S	S	S
Erythromycin	I	I	S

R, Resistant; I, Intermediate; S, Susceptible

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 4: Concentration of lactic acid and acetic acid (mM; mean ± SD) and pH of the supernatants obtained from MRS with cultures.

Strain	pH	Lactic acid (mM)	Acetic acid (mM)
E. faecium strain P1	4.52	90.39 ± 4.24[c]	ND
L. salivarius strain P2	3.84	200.17 ± 12.25[b]	ND
L. agilis strain P3	3.82	220.04 ± 4.98[a]	2.71 ±0.53[a]

ND, non-detectable. The initial pH values of MRS broth were 5.67. n=9.
[a,b,c]Mean with different superscripts within a column are significantly different (P<0.05).

DISCUSSION

In the present study, three isolates of LAB (*L. salivarius* strain P2, *L. agilis* strain P3 and *E. faecium* strain P1) were found to produce CLA under the experimental condition. *Lactobacillus salivarius* strain P2, *L. agilis* strain P3 and *E. faecium* strain P1 produced 21.97, 31.08 and 23.35 µg/mL of CLA in the reaction mixture, respectively. Puniya *et al.* (2008) showed that 4 out of 15 isolates which were isolated from rumen of cattle can produce CLA at concentration of 1.83-10.53 mg/g fat in skim milk after 12 h incubation with 2.5 mg/mL of sunflower oil. *Bifidobacterium pseudolongum* subsp. *pseudolongum* LMG 11595 isolated from chicken feces were shown to produce CLA at 211 µg/mL after 72 h incubation in cysteine-MRS broth containing 0.5 mg/mL LA which (Gorissen *et al.*, 2010). The studies of the ability of bacteria isolated from food, human and other sources to produce CLA were also reported. Previous study showed that six out of twenty two potential probiotic bacteria isolated from commercial fermented milks and other culture collections produced 3.89 to 7.12 µg/mL of CLA after 24 h incubation in M17 or MRS broth supplemented with 1 mg/mL of LA (Rodríguez-Alcalá *et al.*, 2011). Barrett *et al.* (2007) showed that 88 out of 870 isolates isolated from infants and adults had the ability to produce 13 to 380 µg/mL of CLA after 48 h incubation in MRS broth supplemented with 0.5 mg/mL of LA. The production of CLA by the isolates in the present study was ranged between 21.97 to 31.08 µg/mL of reaction mixture, was higher or within the range of the previous reports. However, Kishino *et al.* (2002) reported that 18 out of 250 strains from culture collections could produce 70-341 µg/mL of CLA in potassium phosphate buffer with 4 mg/mL LA within 24-72 h. The production of CLA can be affected by intrinsic characteristics of the bacteria such as linoleate isomerase enzyme activity. Different reaction conditions such as type of substrate, concentration of substrate and incubation time could also affect the production of CLA (Pariza and Yang, 1999). As reported by Kishino *et al.* (2002), the production of CLA could be further enhanced by different physical factors such as the presence of oxygen, pH and temperature. In addition to the concentration of CLA, the type of isomers produced is also important. The main CLA isomers, *c*9, *t*11 CLA and t10, c12 CLA are important in exhibiting beneficial functions to the host (Ogawa *et al.*, 2005). In this study, the isolates produced CLA from LA and they were converted mainly into *c*9, *t*11 CLA and lesser amounts of *t*10, *c*12 CLA. The CLA profile was similar to many studies whereby these two main isomers were produced by LAB (Xu *et al.*, 2004; Ogawa *et al.*, 2005; Lee *et al.*, 2006; Gorissen *et al.*, 2011; Rodríguez-Alcalá *et al.*, 2011).

Isomer of CLA, *c*9, *t*11 was responsible for anti-carcinogenesis (Wang *et al.*, 2006) while *t10, c12* CLA was main isomer responsible for obesity treatment (Park *et al.*, 1999). Both isomers have properties of anti-atherosclerotic (Mitchell *et al.*, 2005) and immune system modulation (Albers *et al.*, 2003). As LAB are commonly used as probiotics, the basic probiotic characteristics of these isolates were determined. The first important criteria is the ability of the strains to tolerate acidic condition. In the gastrointestinal (GIT), the bacteria are required to withstand acidic conditions. For instance, it takes about 2.5 h for food to pass through the alimentary tract of chicken (Duke, 1977). Among the three isolates, *L. salivarius* strain P2 and *L. agilis* strain P3 showed better tolerance to low pH in comparison to *E. faecium* strain P1. About 22.98-53.82% of *L. salivarius* strain P2 and *L. agilis* strain P3 survived at pH 2.5 for 2 h and 83.08-87.95% of *L. salivarius* strain P2 and *L. agilis* strain P3 survived at pH 3.0 for 2 h. Only 0.25% of *E. faecium* strain P1 survived at pH 3.0 for 2 h. The pH tolerance of *L. salivarius* strain P2 and *L. agilis* strain P3 were found to be a superior characteristic. Gaudana *et al.*, (2010) reported that the survival percentage of *Lactobacillus* in acidic buffer pH 2.5 was ranged only from 0.80% to 6.90%. Other reports showed that survival rate of *Lactobacillus* in pH 2.5 were not more than 0.2% (Kim *et al.*, 2007; Perelmuter, 2008).

Apart from pH tolerance, it is also essential that the bacteria to be tolerant towards bile. Gotcheva *et al.*, (2002) demonstrated that 0.3% bile is considered to be a critical bile concentration for screening. In the duodenum, bile salts could destruct cell membranes which consist of lipids and fatty acids (Jin *et al.*, 1998). About 45% of *L. salivarius* strain P2 and *L. agilis* strain P3 could tolerate 0.3% bile for 2 h. However, further exposure to bile affect both isolates negatively as only 0.52-7.59% was found to survive after 24 h incubation. Unlike *L. salivarius* strain P2 and *L. agilis* strain P3, *E. faecium* strain P1 not only survived but propagated in the presence of bile in MRS broth. After 24 h, the survival rate was found to be 186.61%. This could be *E. faecium* strain P1 can assimilate oxgall and used for propagation.

In many cases, microorganisms which were fed to the animals would eventually be excreted to the environment.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Transfer of undesired genes such as antibiotic resistance genes within the microbial population in the environment may occur (Saylers *et al.*, 2004). Therefore, the isolates were tested against 8 commonly used antibiotics in humans and animals. Results showed that *L. salivarius* strain P2 and *L. agilis* strain P3 were resistant to two types of antibiotics tested but *E. faecium* strain P1 showed resistance to 5 types of antibiotics. In most cases, antibiotic resistance genes, with the exception of vancomycin, are harboured in extrachromosomal materials (DeLisle *et al.*, 2003). Instability of plasmids may lead to horizontal transfer of the gene. In the case of probiotics application, this is an undesirable trait and the risk should be reduced.

Probiotics have often been reported to be able to provide protection to the host against infections. In the present study, *L. agilis* strain P3, *L. salivarius* strain P2 and *E. faecium* strain P1 showed antibacterial activity against to *Klebsiella pneumonia* K36, *Serratia marcescens* S913, *Salmonella Enteriditis* IMR S1003/07, *Salmonella typhimurium* S1000, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* 0157:H7, *Proteus mirabilis* P184, *P. vulgaris* P147, MRSA IMR S1228/07B, *Staphylococcus aureus* S244, *S. aureus* IMR S1351, *S. epidermidis* S168, *E. feacalis* E227 and *Listeria monocytogenes* L55. Application of probiotics with antibacterial action could limit growth of pathogen and therefore reduce the risk of food contamination (Chaucheyras-Durand and Durand, 2010).

In general, the antibacterial action of LAB is normally due to the production of bacteriocin or organic acids. However, all the three isolates did not show bacteriocin production (data not shown). Nevertheless, the isolates were observed to produce lactic acid (*L. salivarius* strain P2, 200.17 mM; *L. agilis* strain P3, 220.04 mM; *E. faecium* strain P1, 90.39 mM of lactic acid). Lactic acid has been reported to have the ability to lower the internal pH of the cell which would lead to the collapse of electrochemical proton gradient in certain pathogen (Pan *et al.*, 2009). Martín *et al.* (2006) reported that the LAB in their study produced 71.27 to 131.66 mM lactic acid in MRS broth. Not all lactic acid bacteria can produce acetic acid during fermentation. Low amount of acetic acid, 2.71 mM was detected in *L. agilis* strain P3. Martín *et al.* (2006) reported that *L. salivarius* CECT 5713 produced 11.32 mM acetic acid and *L. rhamnosus* GG with 9 mM acetic acid while *Lactobacillus johnsonii* LA1 and *L. casei imunitass* did not produce any acetic acid. The production of lactic acid or acetic acid in GIT of monogastric can help to create an environment which discourage the growth of pathogen and promote the growth of resident microbiota (Chaucheyras-Durand and Duran, 2010).

At the same time, for the purpose of application, the probiotics should also not cause any negative effects on the host. In the present study, *E. faecium* strain P1 showed α-hemolysis and this indicated that it might be a pathogenic bacterium. The hemolytic activity is strain dependant. Hemolytic activity was not observed in *E. faecium* RM11 (Thirabunyanon *et al.*, 2009). But Ryan and Ray (2004) reported that *E. faecium* are either alpha-hemolytic or with no haemolytic activity. Beta-hemolytic *E. faecium* are rarely found (Vancanneyt *et al.*, 2002). Hemolysis activity is undesirable because it would cause bacteremia and endocarditis to host (Malani *et al.*, 2002). In conclusion, based on the results of the present study, *L. salivarius* strain P2 and *L. agilis* strain P3 were found to be suitable candidates for further evaluation. The ability of the strains to produce CLA, survived in low pH and bile, possess antibacterial activity and did not cause hemolysis made them potential probiotic candidates. However, the CLA production efficiency of the isolates should be optimised and *in vivo* study should be conducted to determine its efficiency.

REFERENCES

Albers, R., van der Wielen, R. P., Brink, E. J., Hendriks, H. F., Dorovska-Toran, V. N. and Mohede, I. C. (2003). Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) isomers on immune fuction in healthy men. *European Journal of Clinical Nutrition* **57, 595-603.**

Asanuma, N., Kawato, M. and Hino, T. (2001). Presence of *Butyrivibrio fibrisolvens* in the digestive tract of dogs and cats, and its contribution to butyrate production. *The Journal of General and Applied Microbiology* **47, 313-319.**

Barrett, E., Ross, R. P., Fitzgerald, G. F. and Stanton, C. (2007). Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. *Applied and Environmental Microbiology* **73, 2333-2337.**

Cepeljnik, T., Zorec, M., Kostanjsek, R., Nekrep, F. V. and Marinsek-Logar, R. (2003). Is *Pseudobutyrivibrio xylanivorans* strain Mz5T suitable as a probiotic? An *in vitro* study. *Folia Microbiologica* **48, 339-345.**

Chaucheyras-Durand, F. and Durand, H. (2010). Probiotics in animal nutrition and health. *Beneficial Microbes* **1, 3-9.**

Chin, S. F., Liu, W., Storkson, J. M., Ha, Y. L. and Pariza, M. W. (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *Journal of Food Composition and Analysis* **5, 185-197.**

Clinical and Laboratory Standards Institute (CLSI) (2012). Performance Standards for Antimicrobial Disk Susceptibility Tests Approved Standard-Eleventh Edition (M02-A11) and Twenty-Second Informational Supplement (M100-S22), Wayne, Pennsylvania.

Dhama, K., Verma, V., Sawant, P. I., Tiwari, R., Veid, R., and Chauhan, R. S. (2011). Application of probiotics in poultry: Enhancing immunity and beneficial effects on production performances and health - A review. *Journal of Immunology and Immunopathology* **13, 1-19.**

DeLisle, S. and Perl, T. M. (2003). Vancomycin-resistant enterococci: A road map on how to prevent the emergence and transmission of antimicrobial resistance. *Chest* **123, 504S-518S.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Duke, G. E. (1977). Avian digestion. *In:* Physiology of Domestic Animals. Duke, G. E. (ed.). 9th edn. Cornell University Press, Ithaca. **pp. 313-320.**

Eyssen, H. and Verhulst, A. (1984). Biotransformation of linoleic acid and bile acids by *Eubacterium lentum*. *Applied and Environmental Microbiology* **47, 39-43.**

Fukuda, S., Furuya, H., Suzuki, Y., Asanuma, N. and Hino, T. (2005). A new strain of *Butyrivibrio fibrisolvens* that has high ability to isomerize linoleic acid to conjugated linoleic acid. *The Journal of General and Applied Microbiology* **51, 105-113.**

Gaudana, S. B., Dhanani, A. S. and Bagchi, T. (2010). Probiotic attributes of *Lactobacillus* strains isolated from food and of human origin. *British Journal of Nutrition* **103, 1620-1628.**

Gorissen, L., Raes, K., Weckx, S., Dannenberger, D., Leroy, F., De Vuyst, L. and De Smet, S. (2010). Production of conjugated linoleic acid and conjugated linolenic acid isomers by *Bifidobacterium* species. *Applied Microbiology and Biotechnology* **87, 2257-2266.**

Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z. and Angelov, A. (2002). Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food Biotechnology* **16, 211-215.**

Gorissen, L., Weckx, S., Vlaeminck, B., Raes, K., De Vuyst, L., De Smet, S. and Leroy, F. (2011). Linoleate isomerase activity occurs in lactic acid bacteria strains and is affected by pH and temperature. *Journal of Applied Microbiology* **111, 593-606.**

Hall, T. A. (1999). Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41, 95-98.**

Holzapfel, W. H. and Schillinger, U. (2002). Introduction to pre- and probiotics. *Food Research International* **35, 109-116.**

Jin, L. Z., Ho, Y. W., Abdullah, N. and Jalaludin, S. (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Letters in Applied Microbiology* **27, 183-185.**

Jin, L. Z., Ho, Y. W., Abdullah, N. and Jalaludin, S. (1996). Effect of adherent *Lactobacillus* spp. on *in vitro* adherence of salmonellae to the intestinal epithelial cells of chicken. *Journal of Applied Bacteriology* **81, 201-206.**

Kennedy, A., Matinez, K., Schmidt, S., Mandrup, S., Lapoint, K. and McIntozh, M. (2010). Anti-obesity mechanisms of action of conjugated linoleic acid. *The Journal of Nutritional Biochemistry* **21, 171-179.**

Kepler, C. R., Hirons, K. P., McNeill, J. J. and Tove, S. B. (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *The Journal of Biological Chemistry* **241, 1350-1354.**

Khanal, R. C. and Olson, K. C. (2004). Factors affecting conjugated linoleic acid (CLA) content in milk, meat, and egg: A review. *Pakistan Journal of Nutrition* **3, 82-98.**

Kim, P. I., Jung, M. Y., Chang, Y. H., Kim, S., Kim, S. J. and Park, Y. H. (2007). Probiotic properties of *Lactobacillus* and *Bifidobacterium* strains isolated from porcine gastrointestinal tract. *Applied Microbiology and Biotechnology* **74, 1103-1111.**

Kim, Y. J., Liu, R. H., Rychlik, J. L. and Russell, J. B. (2002). The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12 isomer of conjugated linoleic acid. *Journal of Applied Microbiology* **92, 976-982.**

Kishino, S., Ogawa, J., Omura, Y., Matsumura, K. and Shimizu, S. (2002). Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. *Journal of the American Oil Chemist' Society* **79, 159-163.**

Koppová, I., Lukás, F. and Kopecný, J. (2006). Effect of fatty acids on growth of conjugated linoleic acid producing bacteria in rumen. *Folia Microbiologica* **51, 291-293.**

Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, K. E., Paek, K. S., Lee, Y. and Park, J. H. (2006). Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochimica et Biophysica Acta* **1761, 736-744.**

Lee, K., Paek, K., Lee, H. Y., Park, J. H. and Lee, Y. (2007). Antiobesity effect of *trans*-10, *cis*-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. *Journal of Applied Microbiology* **103, 1140-1146.**

Macouzet, M., Lee, B. H. and Robert, N. (2009). Production of conjugated linoleic acid by probiotic *Lactobacillus acidophilus* La-5. *Journal of Applied Microbiology* **106, 1886-1891.**

Malani, P. N., Kauffman, C. A. and Zervos, M. J. (2002). Enterococcal disease, epidemiology, and treatment. *In:* The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. Gilmore, M. S., Clewell, D. B., Courvallin, P., Dunny, G. M., Murray, B. E. and Rice, L. B. (eds.). American Society for Microbiology, Washington, **pp. 385-408.**

Martín, R., Jiménez, E., Olivares, M., Marín, M. L., Fernández, L., Xaus, J. and Rodríguez, J. M. (2006). *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. *International Journal of Food Microbiology* **112, 35-43.**

Mitchell, P. L. Langille, M. A., Curiie, D. L. and McLeod, R. S. (2005). Effect of conjugated linoleic avid isomers on lipoproteins and atherosclerosis in the Syrian Golden hamster. *Biochimica et Biophysica Acta* **1734, 269-276.**

Ogawa, J., Kishino, S., Ando, A., Sugimoto, S., Mihara, K. and Shimizu, S. (2005). Production of conjugated fatty acids by lactic acid bacteria. *Journal of Bioscience and Bioengineering* **100, 355-364.**

Oh, D. K., Hong, G. H., Lee, Y., Min, S., Sin, H. S. and Cho, S. K. (2003). Production of conjugated linoleic acid by isolated *Bifidobacterium* strains. *World Journal of Microbiology and Biotechnology* **19, 907-912.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Oh, S., Kim, S. H. and Worobo, R. W. (2000). Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. *Journal of Dairy Science* **83, 2747-2752.**

Pan, X., Chen, F., Wu, T., Tang, H. and Zhao, Z. (2009). The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Food Control* **20, 598-602.**

Pariza, M. W. and Yang, X. Y. (1999). Method of producing conjugated fatty acids. United States Patent, 5856149

Park, Y., Storkson, J. M., Albright, K. J., Liu, W. and Pariza, M. W. (1999). Evidence that the *trans*-10, *cis*-12, isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* **34, 235-341.**

Perelmuter, K., Fraga, M. and Zunino, P. (2008). *In vitro* activity of potential probiotic *Lactobacillus murinus* isolated from the dog. *Journal of Applied Microbiology* **104, 1718-1725.**

Puniya, A. K., Chaitanya, S., Tyagi, A. K., De, S. and Singh, K. (2008). Conjugated linoleic acid producing potential of lactobacilli isolated from the rumen of cattle. *Journal of Industrial Microbiology and Biotechnology* **35, 1223-1228.**

Polan, C. E., McNeill, J. J. and Tove, S. B. (1964). Biohydrogenation of unsaturated fatty acids by rumen bacteria. *Journal of Bacteriology* **88, 1056-1064.**

Rodríguez-Alcalá, L. M., Braga, T., Malcata, F. X., Gomes, A. and Fontecha, J. (2011). Quantitative and qualitative determination of CLA produced by *Bifidobacterium* and lactic acid bacteria by combining spectrophotometric and Ag^{+}-HPLC techniques. *Food Chemistry* **125, 1373-1378.**

Rodríguez-Alonso, P., Fernández-Otero, C., Centeno, J. A. and Garabal, J. I. (2009). Antibiotic resistance in lactic acid bacteria and *Micrococcaceae /Staphylococcaceae* isolates from artisanal raw milk cheeses, and potential implications on cheese making. *Journal of Food Science* **74, M284-M293.**

Romero-Pérez, G. A., Inoue, R., Ushida, K. and Yajima, T. (2013). A rapid method of screening lactic acid bacterial strains for conjugated linoleic acid production. *Bioscience, Biotechnology and Biochemistry* **77, 648-650.**

Ryan, K. J. and Ray, C. G. (2004). Sherris Medical Microbiology, 4th edn. McGraw Hill. New York. **pp. 294-295.**

Saylers, A. A., Gupta, A. and Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology* **12, 412-416.**

***The Poultry Site, Global Poultry Trends 2012- Chicken Consumption Steady in Oceania, Rising in Africa* (2012)**. http://www.thepoultrysite.com/articles/2640/global-poultry-trends-2012-chicken-consumption-steady-in-oceania-rising-in-africa. [Retrieved 17 October 2012]

Thirabunyanon, M., Boonprason, P. and Niansup, P. (2009). Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. *Biotechnology Letters* **31, 571-576.**

Vancanneyt, M., Lombardi, A., Andrighetto, C., Knijff, E., Torriani, S., Björkroth, K. J., Franz, C. M., Foulquié Moreno, M. R., Revets, H., De Vuyst, L., Swings, J., Kersters, K., Dellaglio, F. and Holzapfel, W. H. (2002). Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. *Applied and Environmental Microbiology* **68, 1381-1391.**

Wahle, K. W., Heys, S. D. and Rotondo, D. (2004). Conjugated linoleic acids: Are they beneficial or detrimental to health? *Progress in Lipid Research* **43, 553-587.**

Wall, R., Ross, R. P., Shanahan, F., O'Mahony, L., O'Mahony, C., Coakley, M., Hart, O., Lawlor, P., Quigley, E. M., Kiely, B., Fitzgerald, G. F. and Stanton, C. (2009). Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *The American Journal of Clinical Nutrition* **89, 1393-1401.**

Wallace, R. J., McKain, N., Shingfield, K. J. and Devillard, E. (2007). Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *The Journal of Lipid Research* **48, 2247-2254.**

Wang, L. S., Huang, Y. W., Sugimoto, Y., Liu, S., Chang, H. L., Ye, W., Shu, S. and Lin, Y. C. (2006). Conjugated linoleic acid (CLA) up-regulates the estrogen-regulated cancer suppressor gene, protein tyrosine phosphate gamma (PTPgama), in human breast cells**.** *Anticancer Research* **26, 27-34.**

Xu, S., Boylston, T. D. and Glatz, B. A. (2004). Effect of lipid source on probiotic bacteria and conjugated linoleic acid formation in milk model systems. *Journal of American Oil Chemists' Society* **81, 589-595.**

Zakaria, Z. A., Mat Jais, A. M., Goh, Y. M., Sulaiman, M. R. and Somchit, M. N. (2007). Amino acid and fatty acid composition of an aqueous extract of *Channa striatus* (Haruan) that exhibits antinociceptive activity. *Clinical and Experimental Pharmacology and Physiology* **34, 198-204.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538