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ORIGINAL ARTICLE

Cholesterol homeostasis associated with probiotic supplementation *in vivo*

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cholesterol lowering, fermented foods, lactic acid bacteria, *Lactobacillus*, metabolism, probiotic.

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Abstract

Aims: To determine the mechanism underlying the serum cholesterol reduction effect by probiotics isolated from local fermented tapioca (*Tapai*).
Methods and Results: Lactic acid bacteria strains were isolated and examined for acid tolerance, bile salt resistance and hypocholesterolemic properties. Among the isolates, *Lactobacillus plantarum* TAR4 showed the highest cholesterol reduction ability (48·01%). The focus in the *in vivo* trial was to elucidate the cholesterol balance from findings pertaining to serum cholesterol reduction in rat model fed with high fat diet via oral administration. Rats fed with high-cholesterol diet supplemented with *Lact. plantarum* TAR4 showed significant reduction in serum total cholesterol (29·55%), serum triglyceride (45·31%) and liver triglyceride (23·44%) as compared to high-cholesterol diet (HCD) group. There was a significant increment in faecal triglyceride (45·83%) and faecal total bile acid (384·95%) as compared to HCD group.
Conclusions: The findings showed that probiotic *Lact. plantarum* TAR4 supplementation reduced the absorption of bile acids for enterohepatic recycling and increased the catabolism of cholesterol to bile acids and not by suppressing the rate of cholesterol synthesis.
Significance and Impact of Study: Probiotic supplements could provide a new nonpharmacological alternative to reduce cardiovascular risk factors.

Key Points

- *Lactobacillus plantarum* TAR4 exhibited an excellent tolerance to acid and bile salts.
- *Lactobacillus plantarum* TAR4 was able to assimilate cholesterol *in vitro*.
- *Lactobacillus plantarum* TAR4 had the ability to regulate cholesterol metabolism levels in rats.
- Hypocholesterolemic effect related to cholesterol binding and deconjugation of bile acid by *Lactobacillus plantarum* TAR4.

Introduction

Cardiovascular disease (CVD) has become a great challenge to the human population worldwide (Le and Yang 2018). In 2016, an estimated 17·9 million people died from CVDs, representing 31% of all global deaths. The World Health Organization (WHO) has predicted that by 2030, approximately 23·6 million people around the world will die from CVDs (WHO 2017). Hypercholesterolaemia, an elevated cholesterol level in the blood is a major contributor to the CVDs (Ding *et al.* 2017). Hypercholesterolemia is characterized by an increase in serum total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL) cholesterol (Liu *et al.* 2017). Reduced serum cholesterol has been known as an important goal because it has been reported that a 1% decrease in total serum cholesterol could result in 2–3% reduction in the risk of coronary artery disease (Li *et al.* 2014). However, cholesterol is an indispensable nutrient in human body and thus as a matter of fact, the cholesterol regulation, circulation and the site where the reduced cholesterol is being dispensed is important.

Several studies have shown that the drug therapeutics such as statin, the inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase is essential in the cholesterol metabolic pathway, effectively to reduce the serum cholesterol (Guan *et al.* 2017). However, it is not considered to be an optimal long-term solution due to their associated side effects such as muscle weakness, myalgia, reduced energy, deteriorating hyperglycaemia and increased risk of new-onset diabetes mellitus (Huang *et al.* 2013; Saikia *et al.* 2018).

Other CVD preventative measures that are advocated by healthcare providers include management of modifiable lifestyle risk factors, such as diet, body weight and physical activity. The high mortality rates associated with CVD and the impact on public health expenditure and economic burden due to lost in productivity suggest that these measures are not sufficiently effective and further options are required.

There is increasing evidence with regard to probiotic approach in lowering the cholesterol levels (Lim *et al.* 2017). According to The International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.* 2014). Based on the currently available literature which includes clinical trials, systematic reviews and meta analyses, the consensus panel of ISAPP has summarized that probiotics imparts general benefit of supporting a healthy gut microbiota. Expert panel has defined a species and strain specific effect for the category of probiotic in food or supplement with a specific health claim. Health benefit effect includes cholesterol lowering and bile salt metabolism. Probiotic ability to decrease cholesterol level *in vivo* is strain-specific.

Among the probiotics, lactic acid bacteria (LAB) are regarded as the major group of nonpathogenic bacteria. They are classified as generally recognized as safe microorganisms because of their long and safe use as the starter cultures in the fermented foods (Shehata *et al.* 2016). The considerations of new LAB isolates include tolerance to acid and bile conditions, production of antimicrobial compounds, ability to hydrolyse bile salts and cholesterol lowering potential (Abushelaibi *et al.* 2017). The commonly used LAB for probiotic purposes are *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, *Streptococcus*, *Lactococcus* and *Enterococcus* (Rubio *et al.* 2014; Das *et al.* 2016).

The LAB especially belong to the genera of *Lactobacillus* is best known for its health-promoting effects (Le and Yang 2018). *Lactobacillus* species are part of the commensals in the human gastrointestinal tract (GIT), mouth and female genital tract with the role in preventing the rise of pathogenic microorganisms (Karami *et al.* 2017). The study of Mann and Spoerry (1974) was the first to observe the reduction of serum cholesterol levels in the Massai tribes subjects after the consumption of milk fermented with *Lactobacillus* sp. strain. Therefore, the potential of *Lactobacillus* to lower the serum cholesterol levels *in vivo* has gained much attention recently (Miremadi *et al.* 2014; Liu *et al.* 2017). This could be the prospect of using probiotics as dietary supplements to treat hypercholesterolemia.

Traditional fermented foods could be the readily available resource for LAB with probiotic properties such as cholesterol removal (Patel *et al.* 2014). Lee *et al.* (2011) isolated 12 *Lactobacillus* strains from kimchi and showed the ability to reduce cholesterol level by more than 40% in the *in vitro* test. Albano *et al.* (2018) reported that *Lactobacillus casei* VC199, *Lactobacillus paracasei* ssp. *paracasei* SE160 and VC213, *Lact. plantarum* VS166 and VS513, *Enterococcus faecium* VC223 and *Enterococcus lactis* BT161 isolated from traditional Italian cheeses resulted in cholesterol reduction with the range from 42 to 55% in the medium. Liu *et al.* (2017) also found that the *Lact. plantarum* LP96 isolated from fermented foods reduced the serum TG, serum TC, serum LDL cholesterol, liver TC and liver TG levels significantly in hypercholesterolemic rats. Clinical studies and randomized controlled trials have reported mixed results with regard to the probiotic effect on lipid metabolism (Choi and Chang 2015; Wang *et al.* 2018). Meta-analysis of randomized controlled trials has been carried out to provide a comprehensive evaluation of the studies and the analysis indicates that probiotic supplementations could reduce serum TC. However, the underlying mechanism on the cholesterol balance i.e. modulation of cholesterol esters and lipoprotein leading to the excretion of cholesterol and bile acid or whether it affects the hepatic cholesterol synthesis warrant further studies prior to a conclusive confirmation.

Tapai is a well-known traditional fermented food in Asian countries, especially in Indonesia and Malaysia. It is made typically through the fermentation of cassava, white rice or glutinous rice, and the fermentation process is initiated by a starter culture containing moulds, yeasts and amylolytic bacteria. The preparation of *Tapai* in Malaysia is that the fermentation is carried out in banana leaves (Maslami *et al.* 2018). The development of sweet and sour taste with mild alcoholic flavour in *Tapai* is contributed by the presence of LAB such as *Pediococcus pentosaceus* and *Weissella* sp. in the fermentation. *Tapai* is consumed without cooking, which would not kill any live probiotic LAB; therefore it could be suitable as probiotic carriers (Nuraida 2015). *Tapai* is an excellent source of the potentially beneficial LAB. According to the published literature, few attempts have been made to isolate

LAB from *Tapai* (Kormin *et al.* 2001; Sujaya *et al.* 2001; Adnan and Tan 2007; Suhartatik *et al.* 2014). These studies have attempted to screen and identify the isolated LAB but lack of probiotic characterization including acid and bile tolerance, cholesterol removal ability and antibiotic susceptibility.

To the best of our knowledge, there are no reports on the *in vitro* and *in vivo* hypocholesetrolemic properties of LAB isolated from *Tapai*. Therefore, the aim of this study was to isolate and screen the *Lactobacillus* sp. strains isolated from *Tapai* for probiotic characteristics and *in vitro* cholesterol lowering properties. The effects of isolates on cholesterol levels in hypercholesterolemic or probiotic-supplemented rats were also evaluated to elucidate the cholesterol balance in the body associated with the probiotic treatment by monitoring the serum and liver cholesterol levels, as well as the faecal cholesterol and total bile acid (TBA) concentrations. The value-added milestones from this study include characterization of probiotics to ensure that the isolated LAB strain has the capability to survive under gut condition, comparison of cholesterol lowering effects with a positive control, *Lactobacillus acidophilus* ATCC 4356 which originated from human intestinal tract and has been shown to help reduce cholesterol both *in vitro* (Lin and Chen 2000; Liong and Shah 2005) and *in vivo* (Huang *et al.* 2010, 2014). This is to align to the criteria set under the categorization of live microorganism for human use as defined by expert panel (Hill *et al.* 2014). This report discusses the role of probiotics on cholesterol balance and offers a strain-specific probiotic supplementation as nonpharmacologic alternative for managing CVD risk factors.

Materials and methods

Sample collection

A total of 11 *Tapai* samples were obtained from local market in Kuala Lumpur and Kuantan, Malaysia. The *Tapai* were made from tapioca. All samples were kept in 4°C prior to subsequent analysis.

Isolation of LAB strains

The LAB strains were isolated from *Tapai* (10 g) by blending with 90 ml of bacteriological peptone water. The sample was serial diluted and plated in de Man, Rogosa and Sharpe (MRS) agar medium (Oxoid Ltd, Hampshire, UK). The plates were incubated at 37°C for 48 h under anaerobic condition by placing Oxoid AnaeroGen sachet (Thermo Fisher Scientific, Waltham, MA) in the anaerobic jar. The bacterial colonies on the agar plates were individually selected and streaked on fresh MRS agar plates to obtain the pure cultures (Kormin *et al.* 2001).

The pure cultures were subjected to catalase test using one drop of 3% (v/v) hydrogen peroxide solution. The formation of bubbles indicated the presence of catalase in the bacterial cells. The isolates were then screened for Gram-staining and morphological analysis. The isolates showing Gram-positive and catalase negative and rod-shaped were presumed to be LAB strains, and they were selected for further experiments in this study (Adnan and Tan 2007). The *Lactobacillus acidophilus* ATCC 4356 was purchased from the American Type Culture Collection (ATCC) and used as the positive control.

Tolerance to simulated gastric juice

A 5 ml of MRS broth (Oxoid Ltd) was inoculated at 2% (v/v) with each LAB strain and incubated anaerobically overnight at 37°C. The cultures were centrifuged at 6000 ***g*** for 20 min at 4°C, followed by washing and resuspension in 50 mmol l^{-1} K_2HPO_4 solution twice. A 1 ml of washed cell suspension was harvested by centrifugation at 12,000 ***g*** for 5 min at 4°C and the cell pellet was resuspended in 10 ml of simulated gastric juice. The simulated gastric juice was composed of 5 g of NaCl l^{-1} and 3 g of pepsin l^{-1}. The final pH of the solution was adjusted to pH 3·0 (Wang *et al.* 2012). After 3 h of incubation, the absorbance of the solutions was measured at 600 nm. The strains with more than 10% survival rate in simulated gastric juice of pH 3·0 were chosen for the subsequent assays.

Bile tolerance

The LAB strains were inoculated (2%, v/v) in MRS broth with 0·3, 0·5 and 1·0% (w/v) of oxygall (Difco). The cultures were incubated anaerobically at 37°C for 24 h. The absorbance of the cultures was measured at 560 nm and compared with the control culture (without bile salts) (Wang *et al.* 2012; Kumar and Kumar 2015).

In vitro cholesterol lowering assay

Preparation of cholesterol lowering assay was performed by the procedure of Kim *et al.* (2008). The culture of each LAB strain (10^9 CFU per ml) was inoculated (1%, v/v) in 10 ml of MRS broth supplemented with 0·2% sodium thioglycollate, 0·2% sodium taurocholate and cholesterol. The final concentration of cholesterol in the MRS broth was approximately 100 μg ml^{-1}. An un-inoculated broth was used as a control. The cultures were incubated at 37°C for 24 h under anaerobic condition. The cells were removed by centrifugation at 9000 ***g*** for

15 min at 4°C. The remaining cholesterol in the spent broth was determined by means of calorimetry using *o*-phthalaldehyde method described by Shehata *et al.* (2016). A 1 ml of supernatant was added to 1 ml of 33% (w/v) potassium hydroxide (KOH) and 2 ml of absolute ethanol in a tube and vortexed for 1 min. The tube was heated at 37°C for 15 min. After cooling, 2 ml of distilled water and 3 ml of hexane were added into the tube and vortexed for another 1 min. Approximately 1 ml of hexane layer was pipetted into a new tube and evaporated under nitrogen. Each tube was added with 2 ml of *o*-phthalaldehyde reagent and mixed. Following the addition of 0·5 ml of concentrated sulphuric acid, the mixture was vortexed for 1 min and the absorbance was measured at 550 nm using spectrophotometer after 10 min incubation.

Analysis of cholesterol binding on lactobacilli using scanning electron microscopy

The adhesion of cholesterol particles onto lactobacilli cell was observed by scanning electron microscopy (SEM), modified from the method described by Xiudong *et al.* (2016). The cell pellets obtained were washed five times with sterile saline solution (0·85% NaCl, w/v). The cell pellets were resuspended in 3·5% glutaraldehyde for 2 h at 4°C and dried with 50, 70, 90, 95 and 100% ethanol for 2 min each. The samples were mounted onto a SEM specimen stub, coated with gold and examined under a scanning electron microscope (SNE-3000M; SEC, Suwon, South Korea).

Identification of bacterial isolates using 16S rRNA gene

A total of three isolates were selected for identification using 16S rRNA gene sequencing. The genomic DNA was extracted using the extraction kit Presto™ Mini gDNA Bacteria Kit (Geneaid, New Taipei City, Taiwan). This was followed by PCR amplification of the 16S rRNA gene. The master mix was prepared to a final concentration of 1× PCR buffer (Firepol®), 1·5 mmol l^{-1} of $MgCl_2$ (Firepol®), 0·025 U μl^{-1} of DNA polymerase (Firepol®), 200 μmol l^{-1} of dNTP (Firepol®), 0·2 μmol l^{-1} of 27F primer (AITbiotech, Singapore) and 0·2 μmol l^{-1} of 1429R primer (AITbiotech). PCR amplifications were carried out using the following thermal cycling conditions; initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1·5 min and final extension at 72°C for 10 min for a total of 30 cycles. The amplified DNA was examined by electrophoresis using 1% agarose gel (Vivantis, Malaysia) in 1x TAE buffer at 5 V cm^{-1}. The PCR product was purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid). The purified PCR products were subjected for Sanger sequencing (BioSci and Tech Co. Ltd, New Taipei City, Taiwan). The obtained sequences were then compared with the nucleotide sequences in the NCBI GenBank using the BLAST sequence database.

Nucleotide sequence accession number

Nucleotide sequences obtained in this study have been deposited in the NCBI GenBank database under the accession numbers MH012173, MH012174 and MH012197 for the isolates *Lact. plantarum* TAR4, *Lact. plantarum* TAR7 and *Lact. plantarum* TAR8, respectively.

Preparation of Lactobacillus sp. strains for in vivo experiment

The strains were cultured in MRS broth at 37°C for 18 h in anaerobic condition. The cells were harvested by centrifugation at 8000 ***g*** for 10 min at 4°C. The cell pellets were washed three times using sterile saline solution. After washing, the cell pellets were resuspended in sterile saline solution at approximately 10^9 CFU per ml concentration (Wang *et al.* 2012; Liu *et al.* 2017).

Animal groups and diets

The study was carried out according to the animal care and use guidelines by the Tunku Abdul Rahman University College Animal Ethics Committee by adopting the 3Rs (replacement, refinement and reduction) approach. A total of 30 male Sprague-Dawley rats aged five weeks and weighing 74·00 ± 11·06 g were housed in individually ventilated chambers in GR900 cage with 2GR35 racks (Tecniplast, Buguggiate, Italy); three rats per cage, under controlled room temperature (23 ± 2°C) with about 60-65% relative humidity and maintained on a 12-h light-dark cycle. The rats used in this experiment were obtained from a local breeder (Next Gene Scientific, Selangor, Malaysia). The rats were fed *ad libitum* with commercial pelleted diet in the first week for adaptation. After the adaptation period, the rats were weighted and randomly divided into five groups with the assigned diets as follows: (a) high-cholesterol diet (HCD), (b) high-cholesterol diet supplemented with *Lact. plantarum* TAR4 (HC-4), (c) high-cholesterol diet supplemented with *Lact. acidophilus* ATCC 4356 (HC-LA), (d) normal diet supplemented with saline solution (ND-S) or (e) normal diet (ND). The high-cholesterol diet containing 2% cholesterol (w/w) included 15·9% (w/w) protein, 3·5% fat and 51·7% carbohydrate (TD.07841; Harlan Teklad, Madison, WI). For each day at 09:00 h, the HC-4 and HC-LA groups were orally administered via oral gavage using dosing cannulae, curved stainless steel feeding needle (Harvard Apparatus, Holliston, MA) with 2 ml (10^9 CFU per ml) of cells, and the ND-S group was orally administered an equivalent amount of saline solution for four weeks. The body weight and food consumption of each group were recorded weekly. Corncob was used as the bedding and it was changed every three days. At the end of the study, the rats were fasted for 12 h and euthanized using carbon dioxide.

Serum lipid analysis

The blood samples were obtained from the rats by heart puncture. The blood samples were collected in sterile microcentrifuge tubes and centrifuged at 2000 ***g*** for 20 min at 4°C. The serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) were measured using Cholesterol Fluorometric Assay kit (Cayman), Triglyceride Colorimetric Assay kit (Cayman Chemical, Ann Arbor, MI) and EnzyChrom^{TM} HDL and LDL/VLDL Assay kit (BioAssay Systems, Hayward, CA), respectively (Huang *et al.* 2013).

Liver TC and TG measurement

The rat liver was sampled, rinsed with saline solution, blotted dry and weighed. The liver lipid was extracted using the reagents provided by Triglyceride Colorimetric Assay kit (Cayman) according to the manufacturer's protocol. Briefly, the liver tissue (about 200 mg) was homogenized in 1 ml of NP40 Substitute Assay Reagent containing protease inhibitors. The homogenate was centrifuged at 10 000 ***g*** for 10 min at 4°C. The liver TC and TG concentration in the supernatant were determined using Cholesterol Fluorometric Assay kit (Cayman) and Triglyceride Colorimetric Assay kit (Cayman).

Measurement of faecal TC, TG and TBA

The faecal samples of the rats were collected using disposable wooden chopsticks to avoid contamination with rodent hair or feed and stored at −80°C until analysis. About 50 mg of faecal samples was added with 1 ml of Folch solution (chloroform: methanol = 2 : 1, v/v). The mixture was homogenized and agitated in an orbital shaker for 30 min. The homogenate was centrifuged at 5000 ***g*** for 2 min. The supernatant was collected to analyse for faecal TC, TG and TBA using Cholesterol Fluorometric Assay kit (Cayman), Triglyceride Colorimetric Assay kit (Cayman) and EnzyFluo^{TM} Bile Acid Assay kit (BioAssay Systems), respectively (Lye *et al.* 2017).

Statistical analysis

The statistical data analysis was performed using SPSS (IBM Statistics) ver. 21.0. Analysis of variance (ANOVA) was used to study the significant differences between means with a significant level of $P < 0{\cdot}05$. Means comparison was assessed by Duncan's multiple range test.

Results

Screening of LAB isolates in Tapai

A total of three bacterial strains were isolated from traditional fermented *Tapai*, which are labelled as strain TAR4, TAR7 and TAR8. All the isolated bacteria were considered as LAB as they fit the classification of LAB as rod-shaped, Gram-positive and catalase-negative.

Acid and bile tolerance

The survival rates under simulated gastric juice conditions and the growth in the presence of bile salts of the LAB strains are shown in Table 1. After culturing the strains in the pepsin supplemented simulated gastric juice of pH 3·0 for 3 h, it was shown that all the three strains exhibited over 90% survival rates. The positive control *Lact. acidophilus* ATCC 4356 had high survival rate of 91·62%. Among the tested strains, TAR7 showed significantly ($P < 0{\cdot}05$) higher survival rate of 93·14%, followed by TAR8 and TAR4, which showed 92·14 and 91·90% survival rates, respectively, under simulated gastric juice condition.

Of the three strains, TAR4 showed higher tolerance to 0·3% oxgall with 70·75% of growth, but the growth were drastically decreased to 35·61 and 13·50% in media with 0·5 and 1·0% oxygall, respectively. TAR7 was most sensitive to 0·3% oxgall (49·82%) and showed a decrease in the growth at 0·5 and 1·0% oxgall (31·19 and 16·95%, respectively) after 24 h incubation. The TAR8 showed lower tolerance in 0·3% oxgall (61·66%) as compared to TAR4, and in 0·5 and 1·0% oxgall resulted in reduction in their growth (35·60 and 12·50%, respectively). This result suggests that TAR4 exhibited greater tolerance to pH 3·0 and bile concentration (in particular at 0·3%).

Effects of isolates on in vitro cholesterol lowering

The cholesterol lowering ability of LAB strains was assessed *in vitro* after 24 h of anaerobic growth at 37°C (Fig. 1). All the strains showed the ability to assimilate the cholesterol in the media supplemented with 100 μg ml^{-1} cholesterol, with cholesterol reduction ranging from 38·00 to 48·01%. The strain TAR4 exhibited significantly ($P < 0{\cdot}05$) higher amount of cholesterol

Table 1 Effect of pH and bile concentrations on the viability of LAB strains

Strain	Survival rate (%) under simulated gastric juice	Growth (%) in the presence of oxgall		
		0·3%	0·5%	1·0%
TAR4	91·90^{b}	70·75^{b}	35·61^{b}	13·50^{a}
TAR7	93·14^{a}	49·82^{b}	31·19^{b}	16·95^{a}
TAR8	92·14^{a,b}	61·66^{a,b}	35·60^{b}	12·50^{a}
Lact. acidophilus ATCC 4356	91·62^{b}	62·38^{a,b}	43·28^{a}	10·54^{a}

Values are expressed as means; *n* = 3.

[a,b] Means within the same column followed by different superscript letters are significantly different (*P* < 0·05).

assimilated (48·01%), followed by TAR8, positive control *Lact. acidophilus* ATCC 4356 and TAR7 (45·39, 43·24 and 38·00%, respectively). The negative control strain *E. coli* ATCC 25922 showed the lowest cholesterol reduction (10·33%) among all the tested strains.

SEM was used to analyse the binding of cholesterol to the surface of lactobacilli (Fig. 2). The SEM results showed that the cholesterol particles adhered to the cellular surface of the strains during fermentation (Fig. 2b,d,f, h). It was shown that all the tested strains were capable of assimilating cholesterol from the MRS media.

Identification of LAB isolates

Based on the 16S rRNA gene sequencing, the strain TAR4, TAR7 and TAR8 exhibited 99·93, 99·24 and 99·31% similarity with *Lact. plantarum* strain NWAFU1549, *Lact. plantarum* strain NWAFU1580 and *Lact. plantarum* strain NWAFU1506, respectively (Table 2). Thus, the strain TAR4, TAR7 and TAR8 were designated as *Lact. plantarum* TAR4, *Lact. plantarum* TAR7 and *Lact. plantarum* TAR8, respectively. The sequences were deposited in GenBank with accession number MH012173, MH012174 and MH012197.

Body weight and feed intake

All the rats were generally healthy throughout the feeding trial of four weeks. In the end of the feeding trial, the body weight gain among ND (415·45%), ND-S (371·06%) and HCD (377·09%) groups did not differ significantly (*P*> 0·05), as shown in Table 3. The administration of *Lact. plantarum* TAR4 (HC-4) and *Lact. acidophilus* ATCC 4356 (HC-LA) in high-cholesterol diet groups reduced the body weight gain (334·35 and 355·58%, respectively), but without any significant differences (*P*> 0·05) as compared to HCD group. The total feed intake was ranged between 750·93 g per rat and 782·72 g per rat in ND, HCD, HC-4 and HC-LA groups. The liver weights were not found to significantly (*P* > 0·05) differ among the treatment groups, except for ND-S group which showed significantly (*P* < 0·05) lower liver weight (3·22 g/100g). Sterile saline was used as a vehicle for the administration of LAB strains in rats, and thus ND-S group would act as a control group to determine whether the saline alone causes any effect on the untreated control group (ND).

Serum lipid profiles

The serum LDL cholesterol, HDL cholesterol, total cholesterol and triglycerides levels of the rats in the five groups before and after the treatments are shown in Fig. 3. During adaptation week, the serum LDL cholesterol, HDL

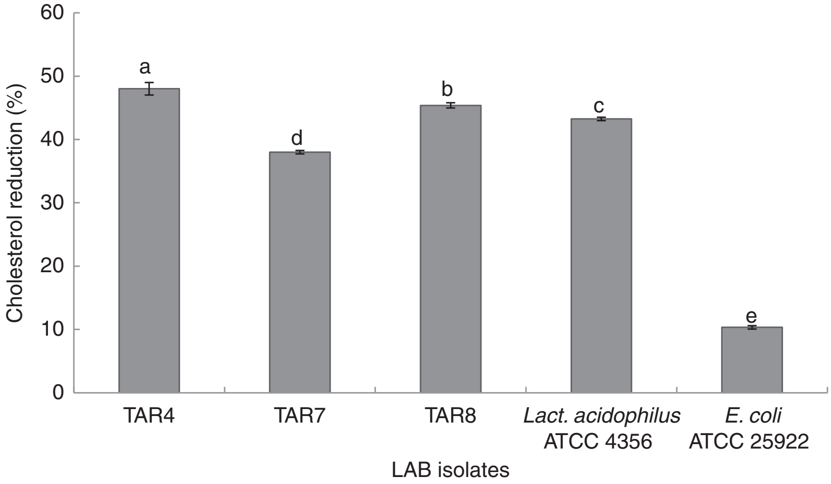

Figure 1 Cholesterol assimilation by isolated LAB strains after 24 h incubation at 37°C under anaerobic condition. Values are expressed as mean ± standard error of means; n = 3. Bars with different lowercase letters indicate significantly different results (*P* < 0·05).

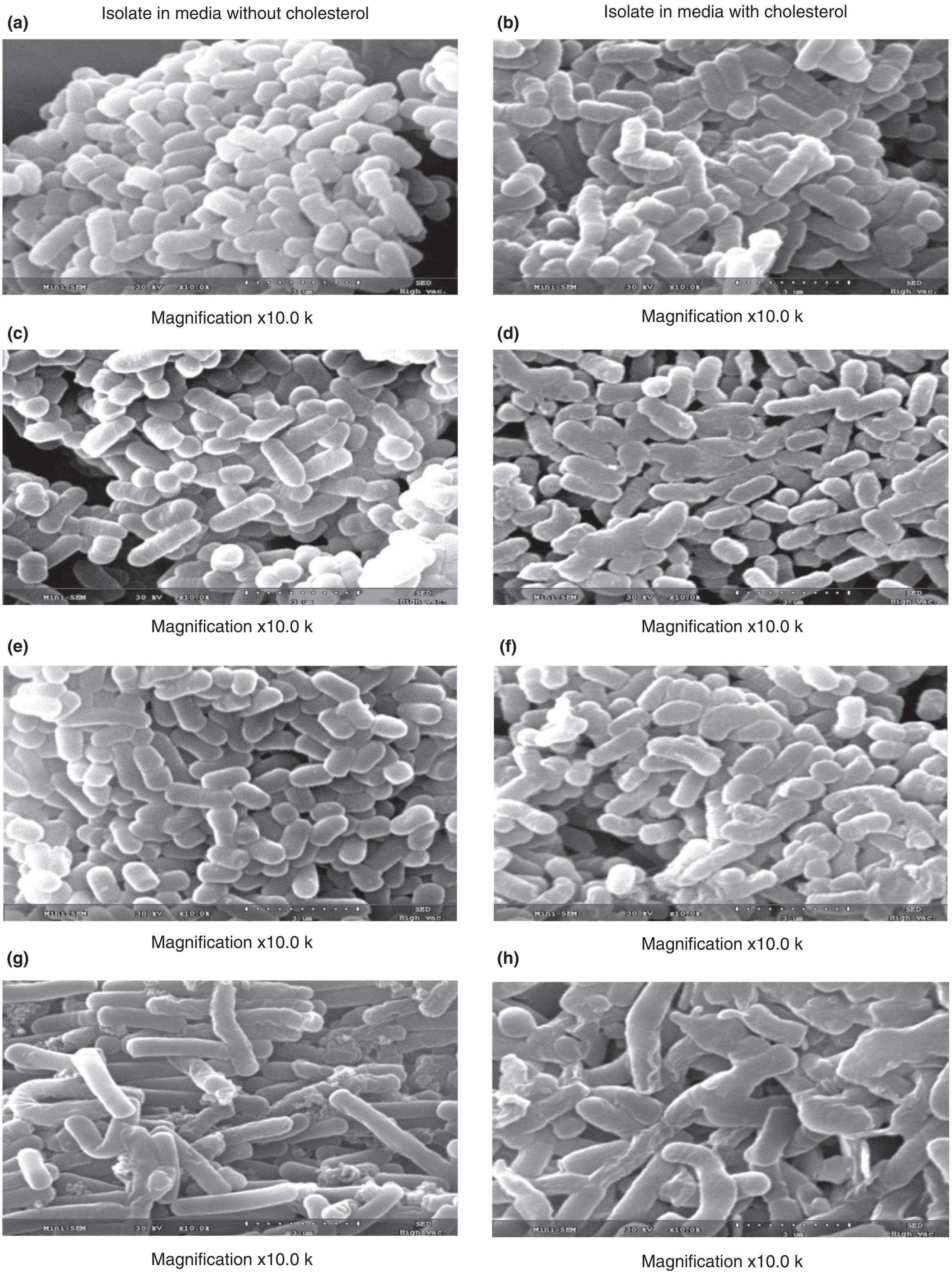
Isolate in media without cholesterol
Isolate in media with cholesterol
(a)
(b)
Mini-SEM 30 kV x10.0k 3 um SED High vac.
Magnification x10.0 k
Magnification x10.0 k
(c)
(d)
Magnification x10.0 k
Magnification x10.0 k
(e)
(f)
Magnification x10.0 k
Magnification x10.0 k
(g)
(h)
Magnification x10.0 k
Magnification x10.0 k

Figure 2 SEM images of (a) *Lactobacillus plantarum* TAR4 grown in MRS broth without cholesterol, (b) *Lact. plantarum* TAR4 grown in MRS broth with cholesterol, (c) *Lact. plantarum* TAR7 grown in MRS broth without cholesterol, (d) *Lact. plantarum* TAR7 grown in MRS broth with cholesterol, (e) *Lact. plantarum* TAR8 grown in MRS broth without cholesterol, (f) *Lact. plantarum* TAR8 grown in MRS broth with cholesterol, (g) *Lact. acidophilus* ATCC 4356 grown in MRS broth without cholesterol and (h) *Lact. acidophilus* ATCC 4356 grown in MRS broth with cholesterol.

Table 2 Similarity values of *Lactobacillus*16S rRNA gene sequences from *Tapai*

16S rRNA phylotype	Size (bp)	Nearest valid taxon	% sequence similarity
TAR4	1445	*Lactobacillus plantarum* strain NWAFU1549	99·93
TAR7	1448	*Lactobacillus plantarum* strain NWAFU1580	99·24
TAR8	1446	*Lactobacillus plantarum* strain NWAFU1506	99·31

cholesterol, TC and TG levels in rats showed no significant differences ($P > 0{\cdot}05$) in all the five treatment groups. The intake of high-cholesterol diet in HCD group resulted in significant ($P < 0{\cdot}05$) increment in serum LDL cholesterol, TC and TG levels as compared to the rats fed with normal diet (ND). This result indicated that the high-cholesterol diets have established the hypercholesterolemic rat model. The supplementation of *Lact. plantarum* TAR4 (HC-4) and *Lact. acidophilus* ATCC 4356 (HC-LA) in high cholesterol diet groups did not show significant reduction ($P > 0{\cdot}05$) in LDL cholesterol levels (35·74 and 32·73 mg dl^{-1}, respectively) and HDL cholesterol levels (40·36 and 32·98 mg dl^{-1}, respectively) compared with HCD group. The serum TC levels in HC-4 and HC-LA groups (55·16 and 63·02 mg dl^{-1}, respectively) were significantly ($P < 0{\cdot}05$) lower than the HCD group (78·30 mg dl^{-1}). The elevated serum TG level in HCD group (106·68 mg dl^{-1}) was significantly decreased ($P < 0{\cdot}05$) by *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356 in HC-4 and HC-LA groups (58·34 and 62·67 mg dl^{-1}, respectively) and showed similar levels with ND and ND-S groups (49·88 and 53·73 mg dl^{-1}, respectively).

Table 3 Body weight gain and liver weight of rats fed with normal or high-cholesterol diet or supplemented with different *Lactobacillus* strains (ND, normal diet; ND-S, normal diet supplemented with saline solution; HCD, high-cholesterol diet; HC-4, high-cholesterol diet supplemented with *Lact. plantarum* TAR4; HC-LA, high-cholesterol diet supplemented with *Lact. acidophilus* ATCC 4356) for the 4-week of feeding trial

	Treatment groups				
Parameters	ND	ND-S	HCD	HC-4	HC-LA
Body weight gain (%)	415·45^{a}	371·06^{a,b}	377·09^{a,b}	334·35^{b}	355·58^{b}
Liver weight (g/100g)	3·78^{a}	3·22^{b}	4·05^{a}	3·73^{a}	3·81^{a}

Values are expressed as means; $n = 5$ (except for ND-S, $n = 3$).

^{a,b}Means within the same row followed by different superscript letters are significantly different ($P < 0{\cdot}05$).

Liver and faecal cholesterol analysis

Liver and faecal lipid contents are shown in Table 4. The liver TC levels in HC-4 and HC-LA groups (48·28 and 58·43 mg dl^{-1}, respectively) did not decrease significantly ($P > 0{\cdot}05$) compared with HCD group (59·77 mg dl^{-1}). The intake of high-cholesterol diets showed significantly higher ($P < 0{\cdot}05$) liver TG level in HCD group (175·09 mg dl^{-1}) as compared with ND and ND-S groups (109·58 and 129·18 mg dl^{-1}, respectively) and the group that received *Lact. plantarum* TAR4 strain (HC-4) and *Lact. acidophilus* ATCC 4356 (HC-LA) decreased their liver TG levels (134·05 and 145·77 mg dl^{-1}, respectively) significantly ($P > 0{\cdot}05$).

Among the treatment groups, similar levels ($P > 0{\cdot}05$) of faecal TC and TG were observed in the adaptation week (week 0), as shown in Table 4. Significant increase ($P < 0{\cdot}05$) in faecal TC level was shown in HCD, HC-4 and HC-LA groups (1499·36, 1625·56 and 1865·99 mg dl^{-1}, respectively) after the treatment as compared with ND and ND-S groups (48·50 and 32·01 mg dl^{-1}, respectively). The HC-LA group (168·01 mg dl^{-1}) showed the highest faecal TG level among the groups, followed by HC-4 (118·87 mg dl^{-1}), HCD (81·51 mg dl^{-1}), ND-S (59·67 mg dl^{-1}) and ND (46·72 mg dl^{-1}) groups, and the differences between the groups were significant ($P < 0{\cdot}05$).

Faecal TBA concentrations

The faecal TBA concentrations of the rats fed different diets are shown in Fig. 4. The TBA levels in faeces in adaptation week (week 0) showed similar levels ($P > 0{\cdot}05$) among the groups. After the 4-week treatments, HC-4 and HC-LA groups demonstrated significant ($P < 0{\cdot}05$) increment in the faecal TBA level (9111·89 mmol l^{-1} and 7386·45 mmol l^{-1}, respectively) as compared with HCD group (1878·93 mmol l^{-1}). The rats fed the normal diets

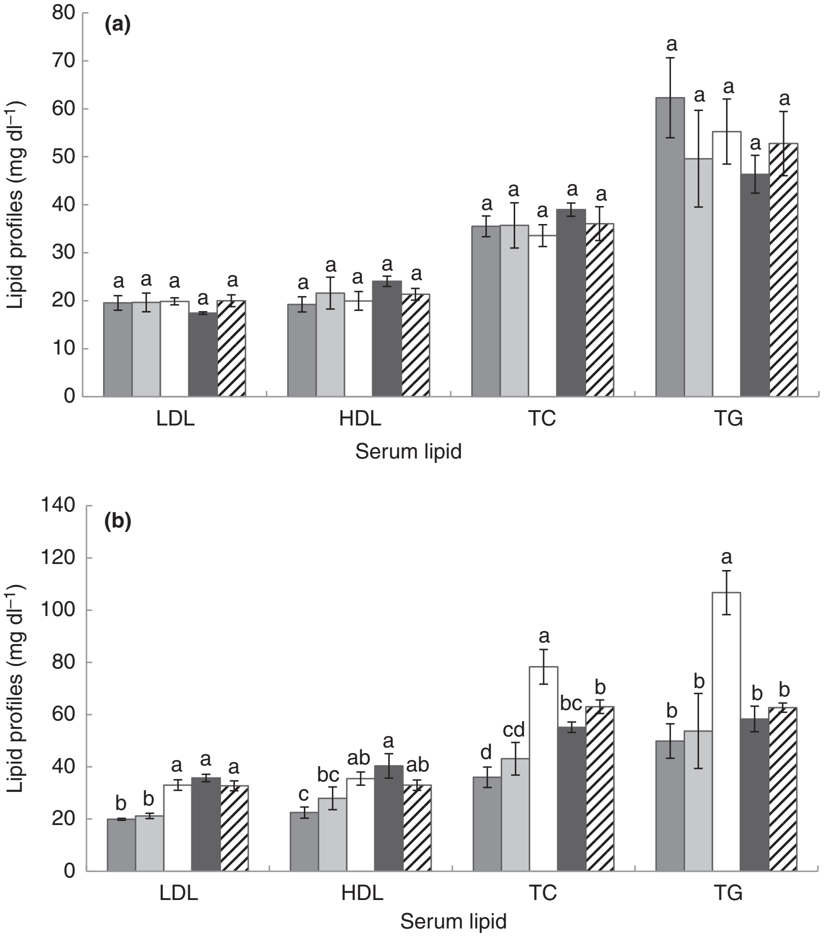

Figure 3 Low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, total cholesterol (TC) and triglyceride (TG) levels in the five groups (▓) ND, normal diet; (▒) ND-S, normal diet supplemented with saline solution; (□) HCD, high-cholesterol diet; (■) HC-4, high-cholesterol diet supplemented with *Lact. plantarum* TAR4; (▨) HC-LA, high-cholesterol diet supplemented with *Lact. acidophilus* ATCC 4356) before (a) and after 4 weeks of treatment (b). The results are expressed as mean ± standard error of means; n = 5 (except for ND-S, n = 3). Mean within same lipid series with different lowercase letters are significantly different ($P < 0{\cdot}05$)

Table 4 The hepatic and faecal lipid content in the five groups (ND, normal diet; ND-S, normal diet supplemented with saline solution; HCD, high-cholesterol diet; HC-4, high-cholesterol diet supplemented with *Lactobacillus plantarum* TAR4; HC-LA, high-cholesterol diet supplemented with *Lactobacillus acidophilus* ATCC 4356) before (week 0) and after feeding trials (week 4)

		Treatment groups				
Parameters	Week	ND	ND-S	HCD	HC-4	HC-LA
Liver TC (mg dl^{-1})	Week 4	35·92^{c}	37·87^{b,c}	59·77^{a}	48·28^{a,b}	58·43^{a}
Liver TG (mg dl^{-1})	Week 4	109·58^{c}	129·18^{b,c}	175·09^{a}	134·05^{b,c}	145·77^{b}
Faecal TC (mg dl^{-1})	Week 0	114·70^{a}	133·55^{a}	123·95^{a}	82·34^{a}	94·91^{a}
	Week 4	48·50^{b}	32·01^{b}	1499·36^{a}	1625·56^{a}	1865·99^{a}
Faecal TG (mg dl^{-1})	Week 0	60·37^{a}	73·75^{a}	72·65^{a}	66·07^{a}	66·39^{a}
	Week 4	46·27^{e}	59·67^{d}	81·51^{c}	118·87^{b}	168·01^{a}

Values are expressed as means; n = 5 (except for ND-S, *n* = 3).

^{a,b,c,d,e}Means within the same row followed by different superscript letters are significantly different ($P < 0{\cdot}05$).

(ND and ND-S) were found to excrete 1814·84 and 1619·52 mmol l^{-1} of TBA in faeces, showing significant ($P < 0{\cdot}05$) lower level than HC-4 and HC-LA groups and no significant difference ($P > 0{\cdot}05$) with HCD group.

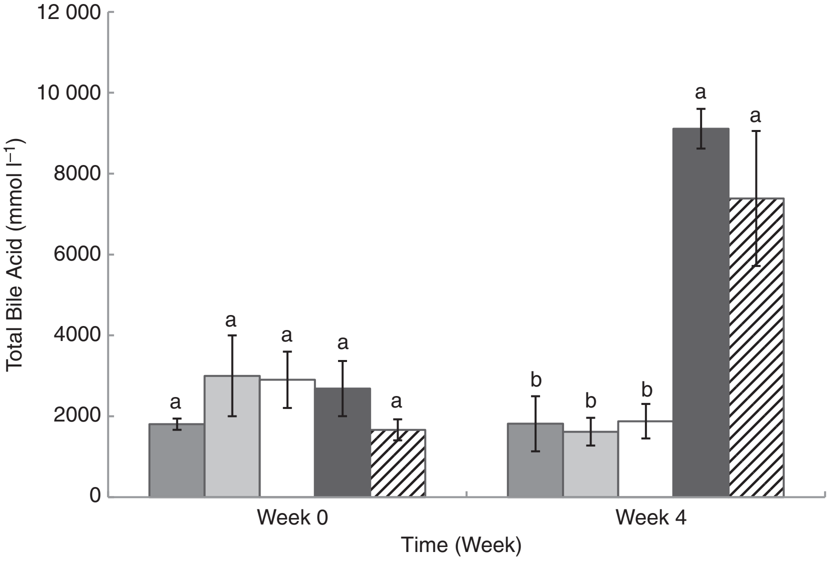

Figure 4 The total bile acid concentrations in faeces of rats fed different diets (■) ND, normal diet; (■) ND-S, normal diet supplemented with saline solution; (□) HCD, high-cholesterol diet; (■) HC-4, high-cholesterol diet supplemented with *Lact. plantarum* TAR4; (▨) HC-LA, high-cholesterol diet supplemented with *Lact. acidophilus* ATCC 4356) before (week 0) and after the feeding trials (week 4). The results are expressed as means ± standard error of means; $n = 5$ (except for ND-S, $n = 3$). Means within the same week with different lowercase letters are significantly different ($P < 0{\cdot}05$)

Discussion

The probiotic lactobacilli are considered as normal inhabitants of the intestinal microflora in humans and animals. They are mostly delivered in a food system and survived passage through the GIT (Kumar *et al.* 2011). In order to exert their beneficial effect on host, they are required to have protective mechanisms to resist the stress conditions of stomach (low pH and digestive enzymes) and small intestine (bile) (Moumita *et al.* 2017). Therefore, acid and bile tolerance is one of the important selection criteria for probiotics (Olejnik *et al.* 2005). One of the factors that determine the survival of bacteria upon the passage through the stomach to the intestine is the pH of the gastric juice (Wang *et al.* 2012). In the present study, the four strains showed high survival rate of over 90% in the simulated gastric juice at pH 3·0 after 3 h of incubation. Similar results were obtained in other studies such as Choi and Chang (2015), who observed 87·1% of survival rate of *Lact. plantarum* EM isolated from kimchi at pH 2·5. In the work of Ding *et al.* (2017), 13 of the 15 isolated LAB strains from fermented Tibetan yak milk showed high survival rates of over 85% after digestion in gastric fluid. The surviving bacteria from the stomach would contact with bile in the small intestine.

The relevant physiological concentrations of bile in human ranged from 0·3 to 0·5%. It is necessary to evaluate the ability of strains to resist the bile salt (Wang *et al.* 2012). The four strains tested in the study exhibited bile tolerance with varying degrees, when the concentrations of oxgall ranged from 0·3 to 1·0%. The tolerance to low pH and bile concentration are strain-specific and this ability is intrinsic. *Lactobacillus plantarum* TAR4 showed higher tolerance due to its intrinsically higher ability to resist stress in acid and bile-containing environment as compared to other isolates. The growth of strains in the presence of high bile concentration indicated that some detoxification mechanisms were included in order to protect them from damage caused by the bile salts (Uroic et al. 2014). Mathara *et al.* (2008) reported that the *Lact. plantarum* strains isolated from fermented milk product *kule naoto* were resistant to 0·3% bile salts and attained 50–84% of growth. Wang *et al.* (2014) also reported that the *Pediococcus acidilactici* B0006, *Lact. plantarum* B0007 and *Lact. plantarum* B0022 isolated from fermented mustard were found to be tolerant to 0·5 and 1·0% bile salts, with growth of over 70%. It was suggested that the resistant mechanism towards acid and bile concentration is strain and species dependent (Abushelaibi *et al.* 2017).

Elevated serum cholesterol levels are generally one of the main factors in the CVD. Current CVD treatment methods include dietary intervention, statins, fibrates, niacin, cholesterol absorption inhibitors and bile acid sequestrants (Jones *et al.* 2013). These approaches have limitations such as side effect associated with long-term usage, intolerance and safety concerns as well as noncompliance to cholesterol lowering medications. Therefore, the wide gap between target and practice currently exists, and additional treatment modalities for serum cholesterol lowering such as the use of probiotics as dietary supplements to treat hypercholesterolemia has gained much attention. Studies have shown that *Lact. plantarum* 91 and *Lact. plantarum* 21 isolated from human faeces assimilated cholesterol from the growth medium in the range from 68·88 to 69·30 μg ml^{-1} (Kumar *et al.* 2012). Miremadi *et al.* (2014) observed that *Bifidobacterium longum* 5022, *Lact. acidophilus* 2404, *Lact. acidophilus* 2410 and *Bifidobacterium bifidum* 5286 from human origin showed cholesterol removal ability at 65, 62, 60 and 52%, respectively. Tulumoglu *et al.* (2013) demonstrated the cholesterol reduction by *Lact. pentosus* originated from human faeces at 76·5% (with oxgall) and 52·4% (without oxgall) after 24 h incubation. In the present study, the four tested strains were all able to effectively assimilate the cholesterol, ranged from 38·00 to 48·01%. Among the isolates, TAR4 showed the highest cholesterol reduction (48·01%) *in vitro* and it was then selected to determine the hypocholesterolemic effect *in vivo*.

The *Lact. acidophilus* ATCC 4356 isolated from human intestinal tract was chosen as the positive control strain in this study as it demonstrated the cholesterol lowering effect both *in vitro* and *in vivo*. Previous studies reported that *Lact. acidophilus* ATCC 4356 was able to reduce cholesterol concentration in the broth containing oxygall, cholic acid and taurocholic acid, respectively (Lin and Chen 2000; Liong and Shah 2005). As reported by Huang *et al.* (2010), the hypercholesterolemic rats given *Lact. acidophilus* ATCC 4356 daily (10^9 CFU per day) for 4 weeks showed reduction of 92·2, 35·5 and 34·2% in serum TC, LDL cholesterol and TG concentrations, respectively. Huang *et al.* (2014) also found that *Lact. acidophilus* ATCC 4356-treated mice exhibited reduction of 37% in the serum TC level as compared to control mice after 16 weeks of treatment. In agreement with the previous investigations, *Lact. acidophilus* ATCC 4356 supplemented diet showed significant reduction ($P < 0·05$) in serum TG level by 41·3% as compared to the control, in the current *in vivo* study.

The rat model with hypercholesterolemia was induced by feeding a 2% high-cholesterol diet. Standard diet with added cholesterol at 2% could induce hypercholesterolemia in rat models without promoting obesity. The feeding of a high-cholesterol diet would increase the body weight in rats (Ding *et al.* 2017). It has been reported that some of the LAB strains can effectively suppress excessive body weight gain in rats. Kang *et al.* (2010) found that the rats fed a high-cholesterol diet supplemented with *Lact. gasseri* BNR17 for 12-week treatment period significantly ($P < 0·05$) decreased the body weight gain at 13·1% compared to the rats fed a high-cholesterol diet. However, the present study showed that the supplementation of *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356 to the high-cholesterol diets did not influence the body weight of rats significantly. These findings indicate that the rats exhibit similar growth patterns in all the treatment groups, and *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356 did not induce significant side effects in the rats. The results obtained in this study is consistent with Park *et al.* (2007) and Wang *et al.* (2009) who reported the similar effects in body weight gain in Sprague Dawley rats following a high-cholesterol diet supplemented with *Lact. acidophilus* ATCC 43121 and *Lact. plantarum* MA2, respectively.

It was well-known that high-cholesterol diet could increase the TC, TG and LDL cholesterol levels in the blood, resulting in an increased risk of cardiovascular events (Li *et al.* 2014). LDL cholesterol is the main component of serum cholesterol. Therefore, lowering of the LDL cholesterol level could be an important factor to reduce the serum total cholesterol levels (Xie *et al.* 2011). The present study indicated that there is no statistical difference ($P > 0·05$) in concentrations of LDL cholesterol in the group fed *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356 when compared with the high-cholesterol diet group. Salaj *et al.* (2013) had reported that *Lact. plantarum* LS/07 and *Lact. plantarum* Biocenol LP96 isolated from rectal human swabs has no effect on the serum LDL levels in hypercholesterolemic Sprague Dawley albino rats.

An increase in HDL concentration is important in controlling the serum cholesterol concentrations as it is capable of removing surplus cholesterol from blood to the liver (Wang *et al.* 2012). In the study, *Lact. plantarum* TAR4 strains did not appear to affect the concentration of HDL cholesterol in rats fed a high-cholesterol diet. The result is in agreement with Pan *et al.* (2011), who reported that the serum HDL cholesterol did not show any significant differences in *Lact. fermentum* SM-7 diets. Abd El-Gawad *et al.* (2005) found that the ingestion of *Bifidobacterium lactis* Bb-12 and *B. longum* Bb-46 showed no significant differences in HDL cholesterol concentration, which is in agreement with present study. However, Chiu *et al.* (2006) reported a reduction in the HDL cholesterol in hamsters fed on high-cholesterol supplemented with *Lact. paracasei* subsp. *paracasei* NTU 101, *Lact. plantarum* NTU 102 and *Lact. acidophilus* BCRC 17010.

Liver plays an important role in regulating the lipid metabolism. The excess lipid in the body is usually deposited in hepatic cells and eventually causes nonalcoholic fatty liver disease (Kim *et al.* 2017; Liu *et al.* 2018).

In the present study, the high-cholesterol diet induced increases in liver TC (66·4% increment) and TG (59·8% increment) as compared to normal diet groups. The administration of *Lact. plantarum* TAR4 to rats fed a high-cholesterol diet effectively reduced the liver TG level by 23·4%. Significant reduction ($P < 0·05$) in total cholesterol levels were observed in the serum (19·5% reduction) and liver (19·2% reduction) of rats in the *Lact. plantarum* TAR4-fed group. The findings support the hypothesis that the deconjugation of bile acids by intestinal microbiota including probiotics via the action of bile salt hydrolase (BSH) increases the levels of deconjugated bile acids to enterohepatic circulation. Increase in deconjugation bile salt decreases cholesterol absorption in the ileal enterocytes. As a result, the cholesterol level in the portal circulation was reduced by means of the decrease in cholesterol absorption in the enterocytes and not being re-distributed between the serum and liver. The result was in agreement with Kim *et al.* (2017), who reported that the hypercholesterolemic rats treated with probiotic mixture of *B. longum* CBG-C11, *B. lactis* CBG-C10, *Bifidobacterium breve* CBG-C2, *Lact. reuteri* CBG-C15 and *Lact. plantarum* CBG-C21 reduced the liver TG levels at 8·34, 16·68 and 36·18% in low ($1·65 \times 10^9$ CFU per kg), medium ($5·5 \times 10^9$ CFU per kg) and high ($1·65 \times 10^{10}$ CFU per kg) doses of probiotic mixtures. Hu *et al.* (2013), reported a significant reduction in liver cholesterol levels in the Sprague Dawley rats fed with *Lact. plantarum* NS5 and *Lact. delbrueckii* subsp. *bulgaricus* NS12.

The mechanisms underlying the hypocholesterolemic effect of LAB have been proposed to involve the inhibition of dietary cholesterol absorption from small intestine (Liu *et al.* 2006; Jeun *et al.* 2010), by binding and assimilation of cholesterol to bacterial cells (Pereira and Gibson 2002; Wang *et al.* 2009; Lye *et al.* 2010) and promotion of faecal bile acid excretion by deconjugation of bile acids (Usman and Hosono 2000; Tok and Aslim 2010; Zheng *et al.* 2013). In the present study, it has been suggested that the *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356 could assimilate the cholesterol through their binding actions (observed via SEM) and increased the excretion of faecal cholesterol by 8·4 and 24·5%, in rat fed with *Lact. plantarum* TAR4 and *Lact. acidophilus* ATCC 4356, respectively. The roughness of the LAB cell wall resulted from the binding of cholesterol particles on the cell surfaces (Kumar and Kumar 2015). The cholesterol was attached to the bacterial cells through a physical phenomenon i.e. the chemical and structural properties of cell wall peptidoglycans containing the amino acids capable of binding cholesterol (Kimoto-Nira *et al.* 2007). The binding of cholesterol onto the cellular surface could inhibit the formation of cholesterol micelle in the intestines. The disrupted micelles could not transport fatty acids to the enterocytes for absorption, leading to reduced cholesterol levels in serum. It was also found that bound-cholesterol are less likely to be absorbed into the small intestine and eventually resulted in an increased excretion of faecal cholesterol and reduced serum and liver cholesterol in rats (Lye *et al.* 2010, 2017).

In the current investigation, significant ($P < 0·05$) increment of TBA was found in the faeces of rats supplemented with *Lact. plantarum* TAR4 (9111·89 mmol l^{-1}) and *Lact. acidophilus* ATCC 4356 (7386·45 mmol l^{-1}) as compared to rats fed a high-cholesterol diet (1878·93 mmol l^{-1}) after 4 weeks of experimental period. The result indicated that the probiotic strains could reduce the cholesterol level in rats due to the deconjugation of bile acid by the BSH activity, and thus promote the bile acid excretion in the faeces. Bile acids are synthesized from cholesterol in the liver through rate limiting enzyme cholesterol 7 α-hydroxylase (CYP7A1). During the normal enterohepatic circulation, about 95% of bile acids are reabsorbed in the distal ileum and transported to the liver (Gérard 2014). Some of the probiotic strains have been found to produce the enzyme BSH that catalyses the hydrolysis of conjugated bile acids. The deconjugation of bile acids formed from the hydrolysis reduces the solubility of bile acid and becomes less reabsorbed from the intestinal lumen, which resulted in excretion of free bile acids in the faeces (Jones *et al.* 2013; Salaj *et al.* 2013; Wahlstrőm *et al.* 2016). Increased excretion of bile acids in the faeces, results in lesser bile acids that are reabsorbed in the ileal enterocytes. Hence, the lower concentration of bile acids that are carried back into portal circulation increases cholesterol catabolism and subsequently leads to an increase of bile acids synthesis as a feedback mechanism regulated by CYP7A1 to maintain cholesterol homeostasis. Therefore, cholesterol level in the body could be reduced through interrupting the enterohepatic circulation and increasing the bile acid biosynthesis (Jones *et al.* 2004; Wahlstrőm *et al.* 2016).

The *Lact. plantarum* TAR4 isolated from *Tapai* showed strong tolerance to low pH and bile salts and higher cholesterol removal ability *in vitro* (48·01%) through binding of cholesterol by LAB. Supplementing rats fed a high-cholesterol diet with *Lact. plantarum* TAR4 (HC-4 group) could effectively reduce the levels in serum TC (29·55% reduction), serum TG (45·31% reduction) and liver TG (23·44% reduction) of rats, and increase the TG (4·83% increment) and total bile acid (385·95% increment) excretion in faeces as compared with HCD group. These results indicated that the *Lact. plantarum* TAR4 has proven as potential probiotic supplement for reducing hypercholesterolemia via binding and assimilation of cholesterol to LAB cells, increase excretion of faecal cholesterol, promotion of faecal bile acid excretion by deconjugation of bile acids and reduced absorption of bile acid in intestine leading to reduced serum and liver cholesterol. The findings provide insights into the cholesterol lowering mechanism due to probiotic LAB supplementation prior to further molecular mechanistic study, probiotic intervention or usage in preventive medicine or even in person-tailored preventive measures.

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Conflict of Interest

The authors declare no conflict of interest.

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