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Original article

The probiotic role of *Lactobacillus plantarum* in reducing risks associated with cardiovascular disease

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Summary A novel strain of *Lactobacillus plantarum* DMDL 9010 (CGMCC No. 5172) was isolated from naturally fermented mustard. The potential cholesterol reduction effects of this strain were investigated using an *in vivo* model. The results showed that *L. plantarum* DMDL 9010 at a dose of 10^9 cells per day significantly reduced ($P \leq 0.05$) serum total cholesterol (TC), low-density lipoprotein cholesterol content (LDL-C) levels and atherosclerosis index (AI) by 23.03%, 28.00% and 34.03%, respectively, while *L. plantarum* DMDL 9010 did not exhibit a significant effect on reducing serum triglycerides (TG) and increasing the serum high-density lipoprotein cholesterol content (HDL-C) in experimental rats ($P > 0.05$). The morphological and pathological changes in the liver illustrated that *L. plantarum* DMDL 9010 protected the rats against hepatocyte steatosis. Additionally, a high dose of *L. plantarum* DMDL 9010 was shown to exhibit a positive cholesterol-lowering effect through decreasing the liver cholesterol (−33.20%) and triglyceride (−40.86%) levels, and increasing significantly ($P \leq 0.05$) faecal cholesterol (+31.07%) and bile acid excretion (+70.18%). The results demonstrated that *L. plantarum* DMDL 9010 acted in a dose-dependent way to decrease serum and total hepatic cholesterol and triglyceride and enhance faecal excretion of bile acids. In conclusion, these findings suggest that *L. plantarum* DMDL 9010 has potential to be explored as a probiotic for hypercholesterolaemic preventive and therapeutic.

Keywords Cholesterol-lowering, fermented mustard, hyperlipidaemia, *Lactobacillus plantarum*, probiotics.

Introduction

Cardiovascular disease (CVD) is one of the main causes of mortality and morbidity worldwide and accounts for 29% of total global deaths (Deaton *et al*., 2011). Epidemiological and clinical studies have shown that hypercholesterolaemia in the blood and diet is a major risk factor for coronary heart disease in humans. It has been shown that a 1% decline in serum cholesterol is associated with a decline of 2–3% in the risk of coronary artery disease (Manson *et al*., 1992). Hypercholesterolaemia is characterised by an increase in serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). One of the quickest treatment approaches to control serum cholesterol and triglycerides is drug administration (Aazmi *et al*., 2015; Richardson & Eggersdorfer, 2015). Due to the costs and side effects of drugs, interest in probiotic therapies to lower serum cholesterol and the risk of the coronary artery disease has been increasing as a convenient method for cholesterol mediation (Richardson & Eggersdorfer, 2015). Biotherapeutics of the genera *Lactobacillus* and *Bifidobacterium* have been examined for lowering the cholesterol level in the serum by means of bile salt hydrolase activity (Liong & Shah, 2005; Kumar *et al*., 2011).

The potential of probiotics to lower serum cholesterol levels *in vivo* has received considerable attention recently with research focused on probiotic lactobacilli (Ha *et al*., 2006; Park *et al*., 2007). Since Shaper *et al*., (1963) and Mann (1974) first observed a hypocholesterolaemic effect of milk fermented by wild-type starters in Maasai tribesmen, the ingestion of probiotic lactic acid bacteria has been viewed as a potentially

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more natural way to decrease serum cholesterol in humans. Several studies have indicated that probiotic lactic acid bacteria have hypocholesterolaemic properties; for instance, long-term consumption of certain fermented dairy products containing sufficient amounts of active lactic acid bacteria can lower serum cholesterol levels (Nguyen *et al*., 2007; Lye *et al*. 2010) and reduce the risk of cardiovascular disease. However, Thompson *et al*. (1982) and Gilliland & Walker (1990) obtained contradictory results and reported that the consumption of yogurt containing *Lactobacillus acidophilus* did not result in the lowering of serum cholesterol in humans. Moreover, the available information of lowering serum cholesterol is mainly limited to the Western strains and other's origin, which may not have the same bioactive functions in Chinese people because of different food habits and gut ecology (Hongpattarakere *et al*., 2012; Jones *et al*., 2014). *Lactobacillus plantarum* DMDL 9010 was previously isolated from a Chinese fermented Mustard (a type of fermented vegetable) by our group (Fei *et al*., 2014). However, its ability of lowering serum cholesterol *in vivo* is unknown. Hence, the lowering serum cholesterol of *Lactobacillus plantarum* DMDL 9010 was focused on in a Sprague Dawley rat model at aim of using the strain as biotherapeutics for high cholesterol in the Chinese population in this study. Rats were used to examine the effects of *L. plantarum* DMDL 9010 on the reduction of serum and hepatic cholesterol levels induced by a hyperlipidaemic diet. Food ingredients have previously been used to mediate the growth of *L. plantarum*, and these have included ingredients such as inulin, algae components, seed oils and phenolic compounds from plant leaves (Barbosa *et al*., 2015; Ben Moumen *et al*., 2015; Shen *et al*., 2015; da Silva Sabo *et al*., 2015 Zhao *et al*., 2015). Moreover, morphological and pathological changes in the liver and the faecal cholesterol and bile acid levels in the rat model were investigated after 70 days.

Materials and methods

Bacterial strains

Lactobacillus plantarum DMDL 9010 was previously isolated from naturally fermented *Mustard*. The fermentation process was as follows: fresh mustard purchased from local market in Guangzhou –> cleaning and drying –> blanching –> adding into 2–4% (m/V) saline –> fermentation at room temperature for 15 days in a sealed bottle –>measurement pH of saline, when the pH was about 3.0–3.5, the fermented saline was collected to screen the microbes (Liu *et al*., 2015). *Lactobacillus plantarum* DMDL 9010 is preserved in China General Microbiological Culture Collection Center with Accession Number CGMCC No. 5172 in 2011. It was first identified as *Lactobacillus pentosus* DMDL 9010 and then determined to be *Lactobacillus plantarum* DMDL 9010 based on sequence analysis of 16S rDNA (accession numbers: KJ917253) and the flanking regions of the *L-ldh1* gene (Fei *et al*., 2014).

Preparation of lyophilised strain powder

The *L. plantarum* DMDL 9010 strain was grown in MRS broth at 37 °C and pH 6.8 for 18 h in a fermentation tank. The cells were harvested by centrifugation at 8000 ***g*** at 4 °C for 15 min, washed twice with sterile double-distilled water and suspended again in 1.5-fold volumes of freeze-drying protective (10% trehalose) solution to improve the bacterial survival rate when frozen. The mixture was prefrozen at −40 °C for 5 h to obtain a homogeneous freezing block on the inner wall of the container and then processed by vacuum freeze-drying for 18–20 h (Zeng *et al*., 2016). The viable cell number of the lyophilised strain powder was 9.30×10^9 cfu g^{-1} using the plate count method in MRS medium.

Animal feeding schedule and grouping

Fifty male Sprague Dawley rats, 56 days of age and weighing approximately 225 g (mean), were obtained from the Laboratory Animal Center (Southern Medical University, Guangdong, China). The rats were fed with a normal diet (including 20% protein, 4.2% fat, and 56% carbohydrates) and housed in cages at 22–24 °C with 55% relative humidity under a 12-h light/dark cycle. The experiment was performed according to the guidelines set by the Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China). The rats were allowed free access to food and water. After adaptation for 7 days, the rats were randomly divided into five groups of 10 each as follows: (i) ND group (control group), normal diet; (ii) HLD group (model group), hyperlipidaemic diet (including 1% cholesterol, 0.2% bile salt, 10% lard and 88.8% normal diet); (iii) HLD-AC group (positive drug control group), hyperlipidaemic diet and atorvastatin calcium (atorvastatin calcium tablets were dissolved in physiological saline and administered by gavage at a dose of 1 mg mL^{-1} day^{-1} per rat); (iv) HLD-HD group (probiotic group), hyperlipidaemic diet with a high dose of lyophilised *L. plantarum* DMDL 9010 powder (the lyophilised powder was dissolved in physiological saline and administered by gavage at a dose of 10^9 cfu/100 g body weight per day (gbw d^{-1})); and (v) HLD-LD group (probiotic group), hyperlipidaemic diet with a low dose of lyophilised *L. plantarum* DMDL 9010 powder (the lyophilised powder was dissolved in physiological saline and administered by gavage at a dose of 10^7 cfu/100 gbw d^{-1}). This study was

carried out in accordance with international regulations on Animal Experiments and was approved by local ethic committee. The animals were fed for a 70-day period. The body weight was recorded every week, and the food intake was recorded daily.

Determination of the serum lipid concentration

At the 28th, 56th and 70th days, blood was collected from the tail vein and enterocoelia after overnight food deprivation. Serum was collected by centrifugation at 3000 ***g*** for 15 min at 4 °C after incubation at room temperature for 1 h. The serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) content were enzymatically measured using a Hitachi 7180 automatic biochemical analyser.

Gross observation and pathological sections of liver tissue

The rats were euthanised after a feeding period of 70 days. The livers were removed immediately, and gross morphology and surface colour were observed and photographed. After weighing, the left lobe of the liver was dehydrated, embedded in paraffin, sliced, haematoxylin–eosin stained (Shackelford *et al*., 2002) and then observed with an LEICA DM5000B optical microscope (Leica Instrument, Germany).

Determination of liver TC and TG content

Liver lipids were extracted according to the method of Folch *et al*. (1957) with a slight modification. A piece of liver (50 mg) was placed in a sample bottle and extracted with chloroform/methanol (v/v, 2:1) in a final volume 20 times the tissue sample and then agitated in an orbital shaker at 37 °C for 30 min. After centrifugation (8000 ***g***, 10 min at 4 °C) and evaporation, the lipid residues were dissolved in isopropanol/Triton X-100 (v/v, 9:1) and distilled water. The liver TC and TG content was measured using enzymatic kits (BioSino Bio-technology and Science Inc, Beijing, China).

Determination of faecal steroid content

Faeces of three consecutive days at 10th week were collected and dried to a constant weight. TC of faeces was extracted with chloroform/methanol (v/v, 2:1) and then incubated at 25 °C for 24 h. After centrifugation (8000 ***g***, 10 min at 4 °C), 0.1 mol L^{-1} potassium hydroxide–ethanol was added to the supernatant and the mixture was saponified at 50 °C for 60 min. The supernatant was evaporated under nitrogen gas, and the residues were dissolved in isopropanol: Triton X-100 (v/v, 9:1) and distilled water. The resulting solution was used to determine the faecal TC content using an enzymatic kit (BioSino Bio-Technology and Science Inc, Beijing, China).

Determination of faecal bile acid content

Bile acids were extracted from the faces according to the method of Carr *et al*. (1993). Briefly, lyophilised faces were weighed (50 mg) and extracted with 10 mL of ethanol at 80 °C. After two extractions, the ethanol was evaporated under nitrogen gas. Subsequently, the residue was dissolved in petroleum. Then, the supernatant was removed and the precipitate was dissolved in ethanol containing 2% Triton X-100. Finally, the supernatant was evaporated under 90 °C for precipitation, dissolved in 10 mL of distilled water, and the resulting solution was used to determine the faecal bile acid content using an enzymatic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

The experimental data are presented as the mean ± standard deviation (SD). The data were analysed by one-way analysis of variance using SPSS (Statistical Package for the Social Sciences) version 19.0 (SPSS, Chicago, IL, USA) followed by a least significant difference (LSD) test. Confidence limits were set at $P < 0.05$.

Results

Body weight and food intake

All rats were generally lively and healthy throughout the feeding period of 70 days. There were no significant differences ($P \geq 0.05$) in body weight gain, food intake and food efficiency among all the groups, while the rats fed on a cholesterol-enriched diet showed a slightly higher body weight gain and food intake in comparison with the rats supplement with normal diet, and the food efficiency (15.02 ± 4.68%) of rats treatment with high dose of DMDL9010 was lower than other groups (Table 1).

Serum lipid analysis

Changes in serum TC (serum total cholesterol), TG (triglyceride), HDL-C (high-density lipoprotein cholesterol) and LDL-C (low-density lipoprotein cholesterol) levels during the experimental period in the groups are shown in Table 2. The TC levels of probiotic treatment groups increased significantly ($P \leq 0.05$) after feeding hypercholesterolaemic diet in comparison with the ND group. A nonsignificant effect of probiotic

Table 1 Food intake and feed efficiency in experimental rats. The animals were fed for a 70-day period. The body weight was recorded every week, and food take was recorded daily. ND, normal diet control; HLD, hyperlipidaemic diet control; HLD-AC, hyperlipidaemic diet and atorvastatin calcium control; HLD-HD, hyperlipidaemic diet containing *L. plantarum* DMDL 9010 of 10^9 cfu/100 g body weight per day; HLD-LD, hyperlipidaemic diet containing *L. plantarum* DMDL 9010 of 10^7 cfu/100 g body weight per day

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g d^{-1})	Food intake (g d^{-1})	Food efficiency* (%)
ND	226.5 ± 16.13[a]	460.0 ± 37.59[a]	3.34 ± 0.68[a]	24.19 ± 4.55[a]	14.05 ± 3.21[a]
HLD	223.7 ± 14.09[a]	491.7 ± 29.40[a]	3.83 ± 0.66[a]	25.61 ± 3.52[a]	15.13 ± 2.95[a]
HLD-AC	229.1 ± 18.38[a]	476.6 ± 30.33[a]	3.54 ± 0.61[a]	25.60 ± 3.33[a]	14.09 ± 3.32[a]
HLD-HD	236.9 ± 15.89[a]	482.6 ± 27.71[a]	3.51 ± 0.77[a]	25.55 ± 3.25[a]	13.89 ± 3.42[a]
HLD-LD	223.4 ± 16.34[a]	490.3 ± 31.60[a]	3.81 ± 0.92[a]	26.36 ± 2.86[a]	15.02 ± 4.68[a]

*Food efficiency, weight gain (g)/feed intake (g)×100.

Values are means ± SD (n = 10 mice).

Means in the same row with different letters differ significantly at $P < 0.05$.

groups was found on TC after 28 days treatments. The TC levels of group supplemented with high dose of *L. plantarum* DMDL 9010 showed significantly decrease by 23.03% compared with the HLD group after 56 days of dietary treatment ($P \leq 0.05$). The group supplement with high dose of *L. plantarum* DMDL 9010 showed a slightly lower TC levels in comparison with the HLD group after 70 days of treatment ($P \geq 0.05$).

An increase in the TG of the HLD group was observed compared to the control group after 28 days, whereas the TG levels of the HLD-AC, HLD-HD and HLD-LD groups were similar to the ND group. TG values of the probiotic groups were slight lower than that in HLD group after 56 and 70 days of treatment, while there was not much difference in the TG levels of the groups.

A decrease was found in HDL-C levels group for hyperlipidaemic diet in comparison with group for normal diet, which means hyperlipidaemic diet will lower HDL-C levels. Although the HDL-C levels of rats fed the hyperlipidaemic diet supplemented with atorvastatin calcium or *L. plantarum* DMDL 9010 were lower than the rats fed the normal diet, it did not show any significant difference.

An increase in the HDL-C content of the HLD group was observed comparing to the ND group after 28 days feeding, whereas there was no significant difference in rats for hyperlipidaemic diet. The HLD group expressed the highest LDL-C content, and the HLD-AC and HLD-HD groups showed significantly lower LDL-C levels compared to the HLD group ($P \leq 0.05$) after 56 and 70 days of treatment of atorvastatin calcium (by 25.37% and 16.00%, respectively) or *L. plantarum* DMDL 9010 (by 37.31% and 28.00%, respectively).

As we can see, there was no significant difference in AI (atherosclerosis index) of all groups before the experiment. The AI was observed throughout the entire experiment period (Table 3). A nonsignificant effect was found on AI after 28 days treatments. The AI of probiotic treatment groups increased significantly ($P \leq 0.05$) after feeding on the hypercholesterolaemic diet in comparison with that recorded with the ND group. However, the AI of the probiotic treatment groups decreased significantly ($P \leq 0.05$) after 56 days and 70 days of treatment as compared with the HLD group. The AI of the dietary treatment groups of rats also differed significantly ($P \leq 0.05$). What's more, the maximum decline in AI was recorded in the HLD-HD group after 70 days (1.57 ± 0.22) of treatment in comparison with the HLD groups (2.38 ± 0.39).

Morphological changes and pathological examination of the liver

Figure 1 depicts the gross morphological changes of the livers of the experimental rats. The livers of the rats in the ND group showed normal morphology with a smooth and dark red surface. Meanwhile, the HLD group exhibited larger volume livers with softer texture, pale yellow surface and greasy appearance. It indicates that the high-fat diet induces hepatic steatosis to some extent. The colour of the livers in the HLD-LD group was pale yellow and similar to the HLD group, while the colour of the rat livers in the HLD-HD group was between the ND and HLD-LD groups. Results indicate that a high dose of *L. plantarum* DMDL 9010 helps to improve hepatic steatosis.

Figure 2 shows the pathological observations of hepatic tissue slices from the experimental rats after haematoxylin–eosin staining. Hepatocytes of the ND group rats possess an integrated, clear and cord-like arrangement without cell steatosis, whereas the HLD group samples have a disordered arrangement and evidence of vesicle steatosis that ranges from moderate to severe. Compared with the HLD group, the quantity of steatotic cells was decreased and the lipid drops of hepatocytes were reduced or absent in the group treated with probiotics. The hepatocyte steatosis in the rat

Table 2 Comparison of the serum lipid levels in experimental rats (mmol L^{-1}) (M±SD, $n = 10$). During the 28th, 56th and 70th days, fasting blood samples were collected from the tail vein and enterocoelia. Serum was collected by centrifugation at 3000 *g* for 15 min at 4 °C after incubation at room temperature for 1 h

	TC (mmol L^{-1})			TG (mmol L^{-1})			HDL-C (mmol L^{-1})			LDL-C (mmol L^{-1})		
	28 day	56 day	70 day	28 day	56 day	70 day	28 day	56 day	70 day	28 day	56 day	70 day
ND	1.18 ± 0.19^{c}	1.18 ± 0.19^{c}	1.18 ± 0.16^{c}	0.88 ± 0.25^{a}	0.72 ± 0.18^{a}	0.79 ± 0.20^{a}	0.63 ± 0.09^{ab}	0.60 ± 0.07^{a}	0.63 ± 0.12^{a}	0.27 ± 0.08^{b}	0.24 ± 0.08^{c}	0.18 ± 0.04^{c}
HLD	1.47 ± 0.23^{ab}	1.65 ± 0.16^{a}	1.59 ± 0.18^{a}	1.03 ± 0.42^{a}	0.75 ± 0.16^{a}	0.82 ± 0.23^{a}	0.68 ± 0.12^{a}	0.55 ± 0.10^{ab}	0.47 ± 0.06^{b}	0.44 ± 0.11^{a}	0.67 ± 0.13^{a}	0.50 ± 0.07^{a}
HLD-AC	1.51 ± 0.32^{a}	1.40 ± 0.26^{abc}	1.40 ± 0.19^{b}	0.85 ± 0.29^{a}	0.88 ± 0.20^{a}	0.80 ± 0.28^{a}	0.64 ± 0.07^{ab}	0.54 ± 0.10^{ab}	0.52 ± 0.10^{b}	0.48 ± 0.18^{a}	0.50 ± 0.14^{b}	0.42 ± 0.12^{bc}
HLD-HD	1.26 ± 0.37^{bc}	1.27 ± 0.30^{bc}	1.36 ± 0.43^{ab}	0.79 ± 0.34^{a}	0.65 ± 0.20^{a}	0.65 ± 0.35^{a}	0.56 ± 0.15^{b}	0.52 ± 0.10^{ab}	0.53 ± 0.11^{b}	0.37 ± 0.15^{ab}	0.42 ± 0.16^{b}	0.36 ± 0.22^{b}
HLD-LD	1.30 ± 0.27^{abc}	1.48 ± 0.27^{ab}	1.50 ± 0.20^{a}	0.77 ± 0.25^{a}	0.71 ± 0.18^{a}	0.70 ± 0.21^{a}	0.61 ± 0.13^{ab}	0.49 ± 0.10^{b}	0.52 ± 0.11^{b}	0.36 ± 0.11^{ab}	0.55 ± 0.28^{ab}	0.49 ± 0.05^{a}

ND, normal diet control; HLD, hyperlipidaemic diet control; HLD-AC, hyperlipidaemic diet and atorvastatin calcium control; HLD-HD, hyperlipidaemic diet containing *L. plantarum* DMDL 9010 of 10^{9} cfu/100 g body weight per day; HLD-LD, hyperlipidaemic diet containing *L. plantarum* DMDL 9010 of 10^{7} cfu/100 g body weight per day.
TC, serum total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
Values are means ± SD ($n = 10$ mice).
Means in the same row with different letters differ significantly at $P < 0.05$.

livers was better repaired by a high dose of *L. plantarum* DMDL 9010.

Liver and faecal lipid analysis

Figure 3 shows the effect of *L. plantarum* DMDL 9010 on the liver lipid content. The hyperlipidaemic diet significantly increased the TC and TG levels of liver compared to normal diet rats ($P \leq 0.05$). After 70 days of feeding, the liver TC and TG levels in HLD group showed the highest levels as 7.68 and 8.42 mg g^{-1}, respectively. The probiotic groups supplemented with *L. plantarum* DMDL 9010 groups showed significant ($P \leq 0.05$) decrease in liver TC levels of 33.20% and 24.48%, respectively, compared with the HLD group after 70 days of dietary treatment. Also, supplementation of the hyperlipidaemic diet with high dose of *L. plantarum* DMDL 9010 led to significant reduction in TG levels when compared with HLD group ($P \leq 0.05$).

Figure 4 shows the effect of *L. plantarum* DMDL 9010 on the faecal lipid and steroid excretion. The total cholesterol and bile acid levels in faces differed significantly among various groups ($P \leq 0.05$). After 70 days of feeding, rats fed with hyperlipidaemic diet had significantly higher TC and bile acid levels of faeces compared to normal diet group ($P \leq 0.05$). The HLD-AC and HLD-HD groups possessed significantly ($P \leq 0.05$) higher faecal TC levels compared with the HLD group, with increases of 18.82% and 25.85%, respectively. Supplementation with the low dose of *L. plantarum* DMDL 9010 led to considerable increases in TC levels in faeces compared with HLD group. In contrast, the level of faecal bile acid in the probiotic group was significantly ($P \leq 0.05$) increased compared to the HLD group, with the highest rate reaching 70.18%.

Discussion

It was well known that hypercholesterolaemic situations are closely linked with an increased risk of atherosclerosis disease (CHD). Reducing of total cholesterol and LDL-cholesterol in the serum of hypercholesterolaemic conditions is very helpful to decrease the risk of cardiovascular disease (CVD). One of the most promising and cost-effective in lowering cholesterol levels in serum is by adjusting diets by means of probiotic interventions. Probiotic lactobacilli are recorded with various health-promoting effects and are recognised as normal components of the intestinal microflora in humans. As a result, probiotics have been paid close attention and are considered as dietary adjuncts in novel probiotic foods. It has been reported that lactic acid bacteria such as in *Lactobacillus acidophilus* (Park *et al.*, 2007), *Lactobacillus plantarum*

Table 3 Effect of *L. plantarum* DMDL 9010 on the atherosclerosis index of experimental rats. The atherosclerosis indices were calculated based on the results of the serum lipid levels at the 0th, 28th, 56th and 70th days of the experimental period.

Group	D0	D28	D56	D70
ND	0.98 ± 0.40^{a}	0.87 ± 0.22^{b}	0.97 ± 0.18^{c}	0.87 ± 0.19^{c}
HLD	1.23 ± 0.42^{a}	1.16 ± 0.30^{ab}	2.00 ± 0.47^{a}	2.38 ± 0.39^{a}
HLD-AC	1.32 ± 0.43^{a}	1.36 ± 0.44^{a}	1.59 ± 0.58^{ab}	1.69 ± 0.77^{b}
HLD-HD	1.38 ± 0.42^{a}	1.25 ± 0.65^{a}	1.44 ± 0.39^{bc}	1.57 ± 0.22^{b}
HLD-LD	1.01 ± 0.49^{a}	1.13 ± 0.28^{ab}	2.02 ± 0.74^{a}	1.88 ± 0.88^{b}

The atherosclerosis index = (total cholesterol-HDL-cholesterol)/HDL-cholesterol.
Values are means ± SD (n = 10 mice).
Means in the same row with different letters differ significantly at $P < 0.05$.

(Kumar *et al*., 2011; Salaj *et al*., 2013), *Lactobacillus paracasei* (Chiu *et al*., 2006), *Lactobacillus reuteri* (Singh *et al*., 2015), *Enterococcus faecium* (Cavallini *et al*., 2009), *Lactobacillus fermentum* (Xie *et al*., 2011) and *Bifidobacterium longum* (Al-Sheraji *et al*., 2012) had the ability to lower serum cholesterol (Table 4). In this study, *Lactobacillus plantarum* DMDL 9010 which was isolated from Chinese fermented *Mustard* showed the ability to lower the cholesterol content *in vitro* (Liu *et al*., 2015). Previous results showed that the removal rate of cholesterol by *L. plantarum* DMDL 9010 in MRS broth reached up to 37.58%. Moreover, *L. plantarum* DMDL 9010 exhibited good acid and bile salt tolerance and bile salt hydrolase activity (data not shown), indicating that the strain could survive the passage through the stomach and reach the small intestine as well as in food (Lee & Salminen, 1995).

A hyperlipidaemic diet supplemented with *L. plantarum* DMDL 9010 did not influence body weight gain, food intake or food efficiency. These findings indicate that animals exhibit similar growth patterns, and thus, *L. plantarum* DMDL 9010 does not induce significant side effects in animals. The results obtained here correlated with the findings of Park *et al*. (2007) and Wang *et al*. (2009) who also used Sprague Dawley rats found similar effects in body weight gain, food intake and food efficiency following a high-cholesterol diet supplemented with *Lactobacillus acidophilus* ATCC 43121 and *Lactobacillus plantarum* MA2. Sprague Dawley rats have not only the advantages of good operability, but also have satisfied repeatability and stability. So, most of the researchers choose male Sprague Dawley rats as animal model (Table 4).

Hypercholesterolaemia is strongly associated with coronary heart disease and arteriosclerosis (Anderson & Gilliland, 1999), and a reduction in TC and LDL-C in hypercholesterolaemic patients can reduce the incidence of cardiovascular disease (Probstfield & Rifkind, 1991). HDL-C, a protective factor for atherosclerosis, can prevent atherosclerosis by transferring cholesterol from the blood, whereas LDL-C, a risk factor for atherosclerosis, causes the accumulation of cholesterol in blood vessels (Lee do *et al*., 2009). The results showed that *L. plantarum* DMDL 9010 at a dose of 10^9 cells per day was effective in significantly reducing ($P \leq 0.05$) serum TC, LDL-C levels and AI by 23.03%, 28.00% and 34.03%, respectively, while

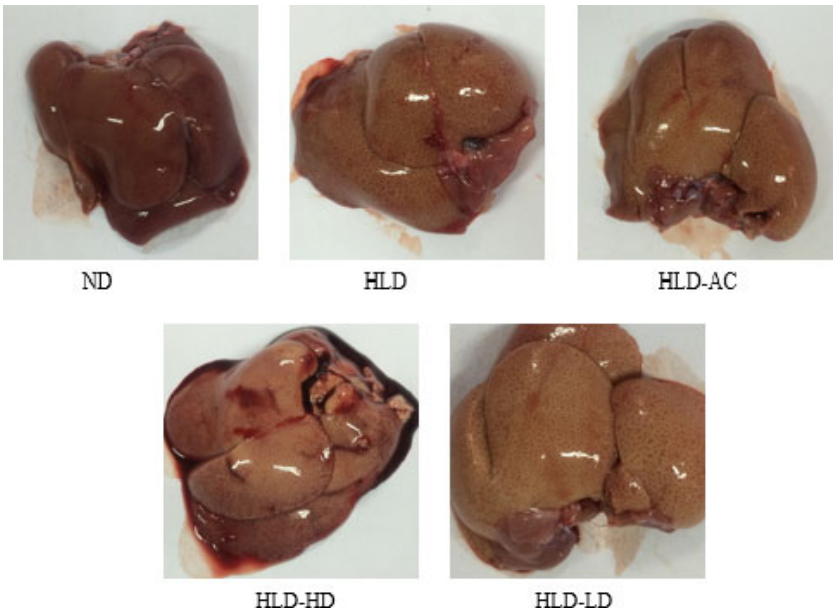

Figure 1 Gross morphological changes in the experimental rat livers. The rats were euthanised after a feeding period of 70 days. The livers were removed immediately, and the gross morphology and surface colour were observed. ND = normal diet (control group), HLD = hyperlipidaemic diet (model group), HLD-AC = hyperlipidaemic diet and atorvastatin calcium (positive drug control group), HLD-HD = hyperlipidaemic diet and a high dose of lyophilised *L. plantarum* DMDL 9010 powder (probiotic group), HLD-LD = hyperlipidaemic diet and a low dose of lyophilised *L. plantarum* DMDL 9010 powder (probiotic group).

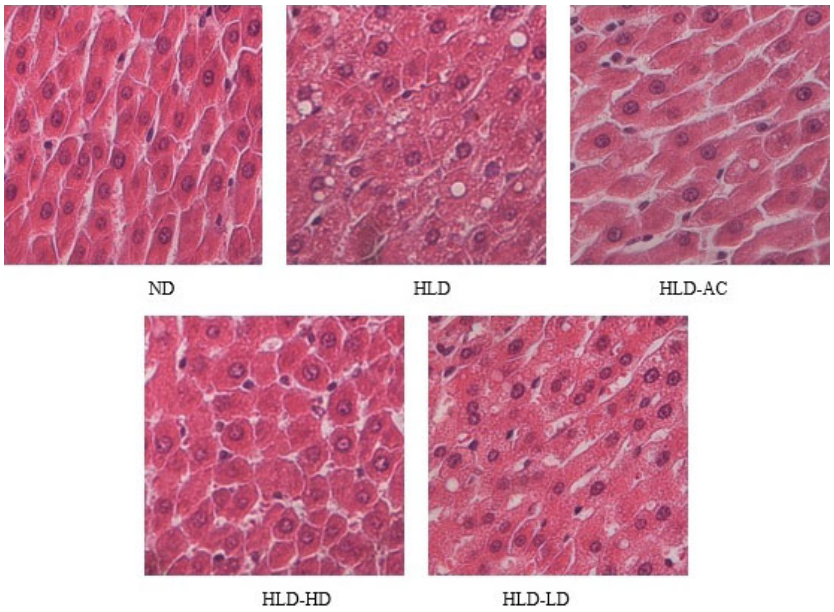

Figure 2 Pathological examination of hepatic tissue slices from experimental rats (original magnification 400×). The rats were euthanised after a feeding period of 70 days. The livers were removed immediately and weighed. Then, the left lobes were dehydrated, embedded in paraffin, sliced, haematoxylin–eosin stained and photographed with an optical microscope (LEICA DM5000B).

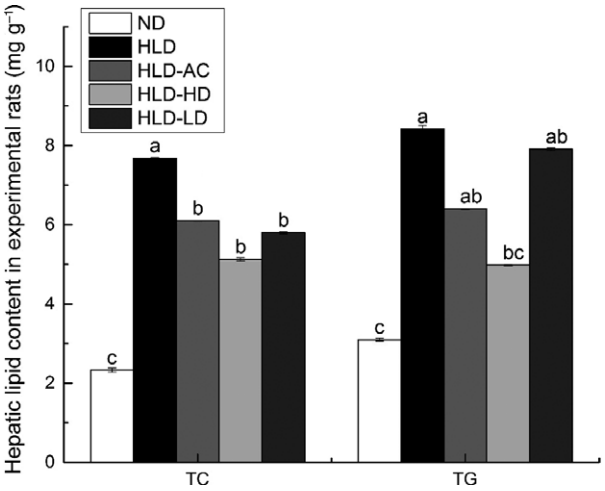

Figure 3 Hepatic lipid content in experimental rats. The rats were euthanised after a feeding period of 70 days. The livers were immediately removed, and the liver TC and TG were extracted and determined.

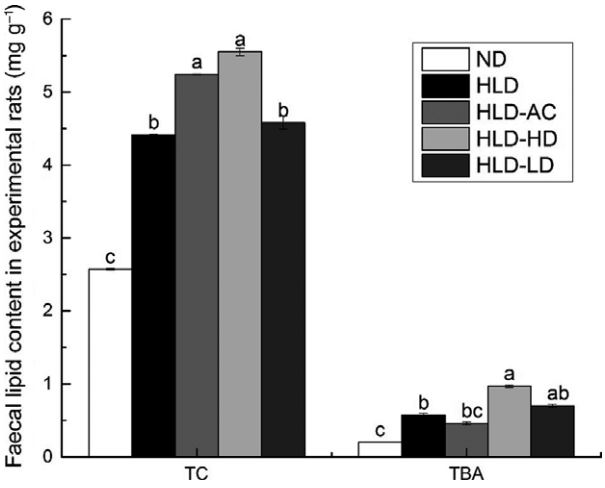

Figure 4 Faecal lipid content in experimental rats. Faeces of three consecutive days at 10th week were collected and dried to a constant weight for determination of total cholesterol and total bile acid content.

L. plantarum DMDL 9010 did not exhibit significant effect on reducing serum TG and increasing the HDL-C content in rats ($P > 0.05$). The results showed that the AI of the probiotic treatment groups decreased significantly ($P \leq 0.05$) after 70 days of treatment as compared with the HLD group. Therefore, it can predicated that *L. plantarum* DMDL 9010 has a positive effect on the reduction of blood lipids in animal experiments based on the Technical Standards for Testing & Assessment of Health Food (Ministry of Health of P.R. CHINA. 2003). These findings are in agreement with Fukushima & Nakano (1996) and Chiu *et al.* (2006), who also reported that *Lactobacillus plantarum, Lactobacillus paracasei* and *Lactobacillus acidophilus* had the ability to decrease serum HDL-C levels in hamsters.

The accurate mechanism of the cholesterol-lowering activity of probiotics has not yet been worked out completely, and probiotics may modify serum cholesterol by four possible mechanisms as follows:

Table 4 Summary of studies on hypocholesterolaemic effects by feeding probiotics in animals

Strains	Strain source	Dose (CFU d^{-1})	Experiment animal	Feeding time/d	TC reduction	HDLC	LDLC	References
L. acidophilus ATCC 43121	UKN	2×10^6	Male Sprague Dawley rats	21	24.39%	NS	ND	Park *et al.* (2007)
L. plantarum Lp91	An Indian gut	1×10^8	Male Sprague Dawley rats	21	23.27%	NS	38.12%	Kumar *et al.* (2011)
L. plantarum LS/07	Rectal human	1.5×10^9	Male Sprague Dawley rats	70	19.87%	NS	NS	Salaj *et al.* (2013)
L. paracasei NTU101	Infant faeces	Free intake	Syrian hamsters	56	26.40%	23.77%	30.46%	Chiu *et al.* (2006)
L. reuteri LR6	Infant faeces	1×10^8	Male Albino rats	60	33.36%	29.31%	68.67%	Singh *et al.* (2015)
E. faecium CRL183	UKN	1×10^8	New Zealand male rabbits	60	NS	42.86%	ND	Cavallini *et al.* (2009)
L. fermentum M1-16	Adults faeces	2×10^9	Male Sprague Dawley rats	42	12.46%	17.32%	NS	Xie *et al.* (2011)
B. longum BB536	UKN	3×10^7	Male Sprague Dawley rats	56	24.64%	NS	37.60%	Al-Sheraji *et al.* (2012)
L. plantarum DMDL9010	Fermented Marstard	1×10^9	Male Sprague Dawley rats	70	23.03%	NS	28.00%	This study

UKN means unknown, NS means not significant, ND means not determined.

1 The assimilation and incorporation of cholesterol enter cellular membranes (Noh *et al.*, 1997), resulting in a decrease in the total cholesterol in enterocytes. Results from scanning electron microscopy proved that cholesterol adhered to the surface of the lactobacilli cells (Lye *et al.*, 2010a). The capability of lactic acid bacteria to assimilate cholesterol has become an important screening criterion *in vitro*.
2 The inhibition of bile acid re-absorption in the small intestine by the production of bile salt hydrolase, which hydrolyses the C-24N-acyl amide bond linking the free bile acid to its amino acid conjugate glycine or taurine, resulting in converting bile salt into free bile acids (Jones *et al.*, 2011). Free bile salts are less water-soluble than conjugated bile salts; thus, they do not participate in enterohepatic circulation and are directly excreted via the faces. Feedback regulation of cholesterol in the liver leads to further decomposition of new bile acids, thereby reducing serum cholesterol levels (Braun *et al.*, 2008; Reynier *et al.* 1981).
3 The inhibition of the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) by the production of ferulic acid (Kim *et al.*, 2003). Statins, inhibitors of HMG-CoA reductase, also play an important role in the competitive inhibition of HMG-CoA reductase, resulting in the reduction of cholesterol levels. Although statins are the primary clinical drugs used to lower cholesterol, they are always associated with unfavourable side effects.
4 Improvement of the activity of cholesterol α-hydroxylase (CYP7A1) (Jeun *et al.*, 2010). Cholesterol is a precursor for the synthesis of bile acid, and CYP7A1 is a rate-limiting enzyme in bile acid biosynthesis (Wertheim *et al.*, 2012). In our studies, more faecal TC and bile acid were detected in the *L. plantarum* DMDL 9010 supplementation group. *L. plantarum* DMDL 9010 may inhibit the reabsorption of bile salts in enterohepatic circulation.

Diet supplementation with cholesterol could result in its accumulation in the liver, leading to an increased atherosclerosis index and the risk of CVD. Supplementation of a hyperlipidaemic diet with *L. plantarum* DMDL 9010 can improve hepatocyte fatty infiltration and decrease the degree of vesicle steatosis to some extent. These findings were in agreement with a previous study (Xie *et al.*, 2011). Morphological and pathological section clearly points to reduction of the lipid drops in hepatocytes (Figs 1 and 2). In the present study, it was shown that total cholesterol and triglyceride levels in liver decreased significantly in the probiotic treatment groups of HLD-HD and HLD-LD (Fig. 3) ($P \leq 0.05$). Higher excretion of total cholesterol and bile acid through faeces in HLD-HD and HLD-LD groups indicated that changes had been brought about by ingested *L. plantarum* DMDL 9010 strain (Fig. 4). Excessive cholesterol is mainly eliminated through conversion to bile acids in liver. The results indicate that *L. plantarum* DMDL 9010 can be effective in inhibiting the accumulation of cholesterol in the liver by increasing the faecal total cholesterol and bile acid content. Lower level of hepatic TC will promote influx of serum cholesterol to liver, thus decrease the serum cholesterol (Table 2). After the cholesterols are converted into bile acids, by means of hepatic portal vessels through enterohepatic circulation, most conjugated bile acids are reabsorbed, while unconjugated ones are excreted into the faeces. If the bile acid re-absorption is blocked by lactic acid bacteria, more bile acid will be excreted in faeces and less will be recycled into liver. Therefore, one possible mechanism for the reduction of cholesterol levels by

L. plantarum DMDL 9010 may be that the re-absorption of bile salts in enterohepatic circulation is inhibited, and free bile acid is excreted into the faeces. Thus, the hyperlipidaemic effect of *L. plantarum* D MDL 9010 may be due to an increase in faecal total cholesterol and bile acids rather than the transfer of cholesterol from the blood to the liver. These findings are in agreement with those of Park *et al.* (2007).

Conclusions

In summary, these results indicate that *Lactobacillus plantarum* DMDL 9010 is a prospective probiotic to lower the risk of cardiovascular disease associated with a cholesterol-enriched diet by reducing serum total cholesterol and LDL-C concentrations and increasing hepatic cholesterol and faecal excretion of bile acid in Sprague Dawley rats. Therefore, we can infer that *L. plantarum* DMDL 9010 is prospective probiotic for the improvement of enterohepatic circulation. Further studies will be required to verify the effects of *L. plantarum* DMDL 9010 in humans and to explore the underlying mechanism behind this observation, for example the transcription factors, cholesterol receptor, key enzymes involved in this lipid homoeostasis.

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Conflicts of interest

The authors declare no conflict of interests.

References

Aazmi, S., Teh, L.K., Ramasamy, K., Rahman, T. & Salleh, M.Z. (2015). Comparison of the anti-obesity and hypocholesterolaemic effects of single *Lactobacillus casei* strain Shirota and probiotic cocktail. *International Journal of Food Science & Technology*, **50**, 1589–1597.

Al-Sheraji, S.H., Ismail, A., Manap, M.Y., Mustafa, S., Yusof, R.M. & Hassan, F.A. (2012). Hypocholesterolaemic effect of yoghurt containing Bifidobacterium pseudocatenulatum G4 or Bifidobacterium longum BB536. *Food Chemistry*, **135**, 356–361.

Anderson, J.W. & Gilliland, S.E. (1999). Effect of fermented milk (yogurt) containing Lactobacillus acidophilus L1 on serum cholesterol in hypercholesterolemic humans. *Journal of the American College of Nutrition*, **18**, 43–50.

Barbosa, J., Borges, S. & Teixeira, P. (2015). *Pediococcus acidilactici* as a potential probiotic to be used in food industry. *International Journal of Food Science & Technology*, **50**, 1151–1157.

Ben Moumen, A., Mansouri, F., Richard, G. *et al.* (2015). Variations in the phytosterol and tocopherol compositions and the oxidative stability in seed oils from four safflower (*Carthamus tinctorius* L) varieties grown in north-eastern Morocco. *International Journal of Food Science & Technology*, **50**, 2264–2270.

Braun, A., Yesilaltay, A., Acton, S. *et al.* (2008). Inhibition of intestinal absorption of cholesterol by ezetimibe or bile acids by SC-435 alters lipoprotein metabolism and extends the lifespan of SR-BI/apoE double knockout mice. *Atherosclerosis*, **198**, 77–84.

Carr, T.P., Andresen, C.J. & Rudel, L.L. (1993). Enzymatic determination of triglyceride, free cholesterol, and total cholesterol in tissue lipid extracts. *Clinical Biochemistry*, **26**, 39–42.

Cavallini, D.C., Bedani, R., Bomdespacho, L.Q., Vendramini, R.C. & Rossi, E.A. (2009). Effects of probiotic bacteria, isoflavones and simvastatin on lipid profile and atherosclerosis in cholesterol-fed rabbits: a randomized double-blind study. *Lipids in Health and Disease*, **8**, 1.

Chiu, C.H., Lu, T.Y., Tseng, Y.Y. & Pan, T.M. (2006). The effects of Lactobacillus-fermented milk on lipid metabolism in hamsters fed on high-cholesterol diet. *Applied Microbiology and Biotechnology*, **71**, 238–245.

Deaton, C., Froelicher, E.S., Wu, L.H., Ho, C., Shishani, K. & Jaarsma, T. (2011). The global burden of cardiovascular disease. *Journal of Cardiovascular Nursing*, **26**, S5–S14.

Fei, Y.T., Liu, D.M., Luo, T.H. *et al.* (2014). Molecular characterization of *Lactobacillus plantarum* DMDL 9010, a strain with efficient nitrite degradation capacity. *PLoS ONE*, **9**, e113792.

Folch, J., Lees, M. & Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, **226**, 497–509.

Fukushima, M. & Nakano, M. (1996). Effects of a mixture of organisms, Lactobacillus acidophilus or Streptococcus faecalis on cholesterol metabolism in rats fed on a fat- and cholesterol-enriched diet. *British Journal of Nutrition*, **76**, 857–867.

Gilliland, S.E. & Walker, D.K. (1990). Factors to consider when selecting a culture of lactobacillus acidophilus as a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science*, **73**, 905–911.

Ha, C.G., Cho, J.K., Chai, Y.G., Ha, Y.A. & Shin, S.H. (2006). Purification and characterization of bile salt hydrolase from *Lactobacillus plantarum* CK 102. *Journal of Microbiology and Biotechnology*, **16**, 1047–1052.

Hongpattarakere, T., Rattanaubon, P. & Buntin, N. (2012). Improvement of freeze-dried *Lactobacillus plantarum* survival using water extracts and crude fibers from food crops. *Food and Bioprocess Technology*, **6**, 1885–1896.

Jeun, J., Kim, S., Cho, S.Y. *et al.* (2010). Hypocholesterolemic effects of *Lactobacillus plantarum* KCTC3928 by increased bile acid excretion in C57BL/6 mice. *Nutrition*, **26**, 321–330.

Jones, M.L., Martoni, C.J., Parent, M. & Prakash, S. (2011). Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *British Journal of Nutrition*, **107**, 1505–1513.

Jones, M.L., Tomaro-Duchesneau, C. & Prakash, S. (2014). The gut microbiome, probiotics, bile acids axis, and human health. *Trends in Microbiology*, **22**, 306–308.

Kim, H.K., Jeong, T.S., Lee, M.K., Park, Y.B. & Choi, M.S. (2003). Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. *Clinica Chimica Acta*, **327**, 129–137.

Kumar, R., Grover, S. & Batish, V.K. (2011). Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague-Dawley rats. *British Journal of Nutrition*, **105**, 561–573.

Lee do, K., Jang, S., Baek, E.H. *et al.* (2009). Lactic acid bacteria affect serum cholesterol levels, harmful fecal enzyme activity, and fecal water content. *Lipids in Health and Disease*, **8**, 21.

Lee, Y.K. & Salminen, S. (1995). The coming of age of probiotics. *Trends in Food Science and Technology*, **6**, 241–245.

Liong, M.T. & Shah, N.P. (2005). Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *Journal of Dairy Science*, **88**, 55–66.

Liu, D.M., Huang, J., Guo, J. *et al.* (2015). Preparation method of Chinese Pickles fermented by mixed lactic acid bacteria: China patent, 201510493977.6.

Lye, H.-S., Rahmat-Ali, G.R. & Liong, M.-T. (2010). Mechanisms of cholesterol removal by lactobacilli under conditions that mimic the human gastrointestinal tract. *International Dairy Journal*, **20**, 169–175.

Mann, G.V. (1974). Studies of a surfactant and cholesteremia in the Maasai. *American Journal of Clinical Nutrition*, **27**, 464–469.

Manson, J.E., Tosteson, H., Ridker, P.M. *et al.* (1992). The primary prevention of myocardial infarction. *New England Journal of Medicine*, **326**, 1406–1416.

Nguyen, T.D.T., Kang, J.H. & Lee, M.S. (2007). Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology*, **113**, 358–361.

Noh, D.O., Kim, S.H. & Gilliland, S.E. (1997). Incorporation of cholesterol into the cellular membrane of Lactobacillus acidophilus ATCC 43121. *Journal of Dairy Science*, **80**, 3107–3113.

Park, Y.H., Kim, J.G., Shin, Y.W., Kim, S.H. & Whang, K.Y. (2007). Effect of dietary inclusion of Lactobacillus acidophilus ATCC 43121 on cholesterol metabolism in rats. *Journal of Microbiology and Biotechnology*, **17**, 655–662.

Probstfield, J.L. & Rifkind, B.M. (1991). The lipid research clinics coronary primary prevention trial: design, results, and implications. *European Journal of Clinical Pharmacology*, **40**(Suppl 1), S69–S75.

Reynier, M.O., Montet, J.C., Gerolami, A., *et al.* (1981). Comparative effects of cholic, chenodeoxycholic, and ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. *Journal of Lipid Research*, **22**, 467–473.

Richardson, D.P. & Eggersdorfer, M. (2015). Opportunities for product innovation using authorised European Union health claims. *International Journal of Food Science & Technology*, **50**, 3–12.

Salaj, R., Stofilova, J., Soltesova, A. *et al.* (2013). The effects of two *Lactobacillus plantarum* strains on rat lipid metabolism receiving a high fat diet. *Scientific World Journal*, **2013**, 135142.

Shackelford, C., Long, G., Wolf, J., Okerberg, C. & Herbert, R. (2002). Qualitative and quantitative analysis of nonneoplastic lesions in toxicology studies. *Toxicologic Pathology*, **30**, 93–96.

Shaper, A.G., Jones, K.W., Jones, M. & Kyobe, J. (1963). Serum lipids in three nomadic tribes of Northern Kenya. *American Journal of Clinical Nutrition*, **13**, 135–146.

Shen, Y., Prinyawiwatkul, W., Lotrakul, P. & Xu, Z. (2015). Comparison of phenolic profiles and antioxidant potentials of the leaves and seeds of Thai holy and sweet basils. *International Journal of Food Science & Technology*, **50**, 1651–1657.

da Silva Sabo, S., Converti, A., Todorov, S.D., Domínguez, J.M. & de Souza Oliveira, R.P. (2015). Effect of inulin on growth and bacteriocin production by *Lactobacillus plantarum* in stationary and shaken cultures. *International Journal of Food Science & Technology*, **50**, 864–870.

Singh, T.P., Malik, R.K., Katkamwar, S.G. & Kaur, G. (2015). Hypocholesterolemic effects of Lactobacillus reuteri LR6 in rats fed on high-cholesterol diet. *International Journal of Food Sciences and Nutrition*, **66**, 71–75.

Thompson, L.U., Jenkins, D.J., Amer, M.A., Reichert, R., Jenkins, A. & Kamulsky, J. (1982). The effect of fermented and unfermented milks on serum cholesterol. *American Journal of Clinical Nutrition*, **36**, 1106–1111.

Wang, Y., Xu, N., Xi, A., Ahmed, Z., Zhang, B. & Bai, X. (2009). Effects of *Lactobacillus plantarum* MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Applied Microbiology and Biotechnology*, **84**, 341–347.

Wertheim, B.C., Smith, J.W., Fang, C., Alberts, D.S., Lance, P. & Thompson, P.A. (2012). Risk modification of colorectal adenoma by CYP7A1 polymorphisms and the role of bile acid metabolism in carcinogenesis. *Cancer Prevention Research (Philadelphia, Pa.)*, **5**, 197–204.

Xie, N., Cui, Y., Yin, Y.N. *et al.* (2011). Effects of two Lactobacillus strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. *BMC Complementary and Alternative Medicine*, **11**, 525–531.

Zeng, X.-A., Zhou, K., D-m, Liu *et al.* (2016). Preparation of fructooligosaccharides using Aspergillus niger 6640 whole-cell as catalyst for bio-transformation. *LWT – Food Science and Technology*, **65**, 1072–1079.

Zhao, C., Wu, Y., Yang, C., Liu, B. & Huang, Y. (2015). Hypotensive, hypoglycaemic and hypolipidaemic effects of bioactive compounds from microalgae and marine micro-organisms. *International Journal of Food Science & Technology*, **50**, 1705–1717.