Cholesterol Removal by *Lactobacillus plantarum* Isolated from Homemade Fermented Cream in Inner Mongolia of China

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Abstract

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A total of 38 strains were initially isolated from Jiaoke, a homemade traditional fermented cream, which was sourced from Inner Mongolia, China, and 6 strains were selected after screening, which was based on their cholesterol removal abilities. The *Lactobacillus plantarum* with the highest level of cholesterol removal from the media was identified using phenotypical characteristics and 16S rRNA sequences, and was named *L. plantarum* KLDS 1.0344. The mechanisms for cholesterol removal involved co-precipitation, assimilation, and degradation of cholesterol by *L. plantarum* KLDS 1.0344. At a level of 8.56 log CFU/ml, *L. plantarum* KLDS 1.0344 survived in pH 2.5 MRS broth for 2 h and exhibited excellent tolerance to 0.3% (w/v) bile. This strain has the most potential in applications as a dietary supplement for lowering human serum cholesterol. A series of tests on animal model and/or clinical tests will be conducted before it can be used in a pharmaceutical application.

Keywords: mechanism; cholesterol; *Lactobacillus*; cream

Jiaoke is a homemade naturally fermented cream in Inner Mongolia of China. The main lactic acid bacteria (LAB) strains isolated from Jiaoke are *Lactobacillus* spp., *Lactococcus* spp., and *Enterococcus* spp. It is no doubt that the fat content is higher than 45% in Jiaoke, and almost all of the cholesterol is present in the fat, but Jiaoke has a lower cholesterol content than other cream products, thus we supposed that some LAB strains from Jiaoke are responsible for its lower cholesterol content.

The literature demonstrated that every 1% reduction in serum cholesterol level, one of the main risk factors for coronary heart diseases, was associated with an estimated 2% to 3% reduction in risk (Manson *et al*. 1992). Since initially reported by Mann and Spoerry (1974) that serum cholesterol levels in men from a tribe of African Maasai decreased after the consumption of large amounts of milk fermented with a wild *Lactobacillus* strain, a number of investigators have examined the effects of *Lactobacillus* strains on cholesterol concentrations (Gilliland *et al*. 1985; Anderson & Gilliland 1999; Liong & Shah 2005; Nguyen *et al*. 2007; Mathara *et al*. 2008). Various studies have shown that some LAB strains could lower cholesterol content (Anderson & Gilliland 1999; Sanders 2000; Jones *et al*. 2004; Adebawo *et al*. 2008; Sridevi *et al*. 2009),

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but the exact mechanisms of serum cholesterol reduction by LAB strains are unclear. Some strains of *L. acidophilus* were found to secrete bile salt hydrolase (BSH; EC 3.5.1.24) which catalysed the hydrolysis of glycine- or taurine-conjugated bile salts into amino acid residues and free bile salts (Corzo & Gilliland 1999). The latter is less soluble than the conjugated bile salts, which results in a lower absorption in the intestinal lumen. Deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids needed to replace those that have escaped the enterohepatic circulation (Reynier *et al*. 1981). Another mechanism suggested for cholesterol reduction resides in that *L. acidophilus* incorporates some of the cholesterol into cellular membranes, and that results in cholesterol being less available for the absorption from the intestine into the blood (Noh *et al*. 1997). However, this was debated, as experiments using the membrane isolated from strains of *L. casei* grown in broth containing cholesterol revealed no measurable incorporation of cholesterol (Brashears *et al*. 1998). Another hypothesis that cholesterol removal may be related to the assimilation of cholesterol was derived from the observation that a significant amount of cholesterol (52%) was retained in the cells of *L. amylovorus* (Grill *et al*. 2000).

The objectives of this study are the assessment *in vitro* of the cholesterol removal ability of *Lactobacillus* strains isolated from Jiaoke, and the evaluation of the possible mechanisms of cholesterol removal including the desirable properties of acid and bile tolerance.

MATERIAL AND METHODS

Collection of samples. 25 samples of Jiaoke made from unpasteurised bovine milk were collected from different areas of Inner Mongolia, China. The production processes were as follows. Milk was placed in a container, such as an iron basin or plastic pail, and then naturally fermented for periods, varying in time from one to eight days. After natural coagulation of the milk, the upper fluid layer was collected and considered as Jiaoke which is a homemade indigenous fermented cream. Some batches were filtered by gauze. The samples were taken at different temperatures, ranging from 15°C to 20°C. They were yellow or white in colours, showed acidic smell and appeared as viscous, oily fluids or solid curd-like substances. The samples were stored in sterilised plastic bottles, kept on ice and transported to the laboratory for analysis.

***Isolation of** Lactobacillus*. A total of 38 strains, suspected to be LAB, from 25 samples were selected by observing the colony appearance, catalase reaction, Gram staining test, and the appearance of a transparent circle on de Man, Ragosa, and Sharpe (MRS) agar supplemented with 0.75% (w/v) $CaCO_3$. The selected LAB strains were restreaked on MRS agar and examined for purity. The isolates were stored at 4°C in MRS agar or freeze-dried and stored at –20°C.

***Screening of** Lactobacillus* **with cholesterol-lowering capacity**. Freshly prepared MRS-THIO broth (MRS broth with 0.2% (w/v) sodium thioglycollate) was supplemented with 0.3% (w/v) oxgall as a bile salt. Sodium thioglycollate was used as an oxygen scavenger (Usman & Hosono 1999). Alcohol-soluble cholesterol was sterilised by filtration (0.22 µm-pore-size filter) and added to the broth at a final concentration of 100 µg/ml. The broths containing each strain at 1% were anaerobically incubated at 37°C in a Forma Anaerobic System (Thermo Electron Corporation, Waltham, USA) for 24 hours.

The cells were removed from the culture broth by centrifugation at 12 000 g and 4°C. For 10 min the supernatant fluid was saved, and the pellets were washed three times with a volume of MRS-THIO broth with 0.3% oxgall equal to that of the original broth. After each washing, the suspension was centrifuged at 12 000 g and 4°C for 10 min and the three supernatants were combined and called washing fluid. The cells of the final pellets were resuspended in MRS-THIO broth containing 0.3% oxgall plus lysozyme at a final concentration of 2 mg/ml, and placed in a water-bath at 37°C for 30 min, and then immediately placed on ice and treated with supersonic wave (SONICS) for 1 hour. The fragmented-cells solution was saved by supplementing with MRS-THIO broth with 0.3% oxgall to the original volume and centrifuging at 12 000 g and 4°C for 10 minutes. In all fractions the cholesterol concentration was determined using a colorimetric method slightly modified in volume as described by Rudel and Morris (1973), taking MRS-THIO broth with 0.3% oxgall reading as the standard for comparison.

Cholesterol removal (CR) was calculated following the equation: CR = (C–C')/C, where C and C' were the concentrations of cholesterol present in the supernatants of not-inoculated and inoculated

LAB strains, respectively. The cholesterol degradation ratio (CDR) was calculated following the equation: CDR = [C – (C1 + C2+ C3)]/C, where C was the concentration of initial cholesterol, and C1, C2, and C3 were the concentrations of cholesterol in the supernatant fluid, washing fluid, and fragmented-cells solution, respectively.

Acid tolerance. Testing the acid tolerance was carried out as described by Liong and Shah (2005), but MRS-THIO broth was adjusted to pH 2.5 with HCl. After the selected strains had been subcultured at least over three generations, the cells were saved from the culture broth by centrifugation at 12 000 g and 4°C for 10 min then suspended in pH 2.5 MRS-THIO broth and incubated anaerobically for 2 h at 37°C. A 1 ml sample was taken every 30 min during 2 h, and 10-fold serial dilutions were made using phosphate buffer (Na_2HPO_4/NaH_2PO_4 buffer 100 mmol/l, pH 7.0). The samples were plated onto MRS agar and incubated anaerobically at 37°C for 24 hours. The colony counts of the time-interval samples were determined as acid tolerance.

Bile tolerance. Bile tolerance, using oxgall as the bile source, was conducted according to the method of Gilliland and Walker (1990). Briefly, MRS-THIO broth either with 0.3% (w/v) of oxgall or without oxgall for control was inoculated with each strain and incubated anaerobically at 37°C. The bacterial growth was monitored by measuring the absorbance with a spectrophotometer (DU 800, Beckman Coulter, Los Angeles, USA) at 620 nm at hourly intervals for 7 h to 8 hours. The absorbance values obtained were plotted against the incubation time, and the bile tolerance of each strain was estimated based on the time required for the absorbance value to increase by 0.3 units. Each strains result was replicated twice.

Identification of *Lactobacillus.* The only isolated KLDS 1.0344 that showed superior properties as a probiotic among the various strains selected was characterised and identified using standard biochemical tests mentioned in Bergey's Manual of Determinative Bacteriology (Buchanna & Gibbons 1984) and then confirmed by amplification and sequencing of the 16S rRNA gene (Woo *et al.* 2003). The sequence obtained was analysed using an online Blastn alignment tool for the comparison with the sequences in the GeneBank.

Statistical analysis. The results are presented as mean value ± standard deviation (SD). The data analysis was carried out with MS Excel 2003 and SAS 9.1.3 statistical program.

RESULTS

Screening of strains with cholesterol-lowering

A total of 38 strains suspected to be LAB from 25 samples of Jiaoke were selected based on the rod shape, positive Gram reactions, and catalase absence. Six strains with higher cholesterol removal ratio (Table 1) were chosen for further investigation.

Acid tolerance of *Lactobacillus*

The effect of acid on the viability of LAB isolate is shown in Table 2. Some strains showed strong tolerance towards pH 2.5 for 2 hours. The most acid-tolerant strain KLDS 1.0344 exposed to pH 2.5 for 2 h showed a slight decrease in the cell viability from 9.36 to 8.56 log CFU/ml, while the most acid-sensitive strains, KLDS 1.0343, KLDS 1.0374, and KLDS 1.0381, showed no viable bacteria after 2 h of incubation.

Bile tolerance of *Lactobacillus*

The effect of oxgall on the growth of LAB isolate is presented in Table 3. The growth of *Lactobacillus* in MRS-THIO broth without bile was used as control. All tested strains grew better in MRS-THIO broth containing oxgall, showing various

Table 1. Cholesterol removal ability of lactic acid bacteria

Strains	CR (%)*
KLDS 1.0343	23.55 ± 1.98^{c}
KLDS 1.0344	54.08 ± 1.36^{a}
KLDS 1.0357	25.85 ± 4.49^{c}
KLDS 1.0365	22.60 ± 3.84^{c}
KLDS 1.0374	24.03 ± 2.48^{c}
KLDS 1.0381	36.55 ± 1.02^{b}

^{abc}means in the same row followed by different superscript letters are significantly different ($P < 0.05$)

*Results are expressed as mean ± SD; the amount of cholesterol lost from supernatant fluid is expressed as a percentage of the initial concentration of cholesterol in the medium; each data point is the average of 2 repeated measurements from 3 independently replicated experiments

Table 2. Effect of pH 2.5 on viability of lactic acid bacteria

Strains	Viable count (log CFU/ml)*				
	0 min	30 min	60 min	90 min	120 min
KLDS 1.0343	9.21 ± 0.03^{a}	8.99 ± 0.09^{a}	4.95 ± 0.20^{b}	3.65 ± 0.19^{c}	0^{d}
KLDS 1.0344	9.36 ± 0.15^{a}	9.42 ± 0.09^{a}	9.30 ± 0.01^{ab}	9.10 ± 0.02^{b}	8.56 ± 0.04^{c}
KLDS 1.0357	9.27 ± 0.04^{a}	9.23 ± 0.07^{a}	9.03 ± 0.08^{b}	7.19 ± 0.03^{c}	3.31 ± 0.07^{d}
KLDS 1.0365	9.49 ± 0.02^{a}	9.56 ± 0.07^{a}	9.16 ± 0.01^{b}	5.25 ± 0.01^{c}	1.77 ± 0.10^{d}
KLDS 1.0374	9.27 ± 0.08^{a}	8.92 ± 0.01^{b}	8.82 ± 0.13^{b}	4.42 ± 0.03^{c}	0^{d}
KLDS 1.0381	9.50 ± 0.01^{a}	9.17 ± 0.09^{b}	7.77 ± 0.25^{c}	3.46 ± 0.02^{d}	0^{e}

^{abcd}means in the same row followed by different superscript letters are significantly different ($P < 0.05$)

*Results are expressed as mean ± SD; each data point is the average of 2 repeated measurements from 2 independently replicated experiments

levels of tolerance towards bile. KLDS 1.0344 grew fastest in the presence of oxgall among the six strains, whereas KLDS 1.0357 showed the slowest growth.

Identification of *Lactobacillus*

The strain KLDS 1.0344 was characterised by standard biochemical tests. KLDS 1.0344 was non-sporulating, Gram-positive, catalase- and nitrite reductase-negative. The strain failed to hydrolyse gelatin and produce gas from glucose. It grew in MRS broth at 15°C, but not at 45°C, and produced acid from melibiose, cellobiose, sucrose, and maltose, but not from xylose and arabinose. 16S rRNA gene analysis of the strain KLDS 1.0344 showed 99% similarities to that of *L. plantarum* strain WCFS1 (GenBank accession No. AL935260). Therefore, the strain KLDS 1.0344 was identified as *L. plantarum*.

Mechanism of cholesterol removal by *L. plantarum* KLDS 1.0344

In order to ensure the mechanism of cholesterol removal from the media, *L. plantarum* KLDS 1.0344 was cultured in MRS-THIO broth containing cholesterol plus oxgall (Table 4). The results revealed that in the presence of *L. plantarum* KLDS 1.0344, 54.08% of cholesterol disappeared from the supernatant fluids. Partly, cholesterol was precipitated and resolubilised in the washing fluids (18.57%), another part of cholesterol (15.69%) was retained in the cells, and yet another

Table 3. Effect of bile on the growth rate of lactic acid bacteria

Strains	Time (h)*	
	MRS-THIO broth	MRS-THIO broth + 0.3% oxgall
KLDS 1.0343	3.52 ± 0.16^{cB}	4.48 ± 0.18^{cA}
KLDS 1.0344	4.23 ± 0.11^{bA}	4.47 ± 0.21^{cA}
KLDS 1.0357	4.65 ± 0.33^{abB}	5.90 ± 0.11^{aA}
KLDS 1.0365	3.52 ± 0.23^{cB}	5.53 ± 0.33^{abA}
KLDS 1.0374	4.97 ± 0.24^{aA}	5.08 ± 0.34^{bcA}
KLDS 1.0381	4.23 ± 0.40^{bA}	4.98 ± 0.31^{bcA}

^{abc}means in the same column followed by different lowercase letters are significantly different ($P < 0.05$)

^{ABC}means in the same row followed by different uppercase letters are significantly different ($P < 0.05$)

*Time (h) required for absorbance at 620 nm to increase by 0.3 units in each medium; results are expressed as mean ± SD; each data point is the average of 2 repeated measurements from 2 independently replicated experiments

Table 4. Cholesterol removal ability of lactic acid bacteria*

Strains	Cholesterol (%)**			CDR (%)***
	supernatant fluid	washing fluid	fragmentised-cells solution	
KLDS 1.0344	45.92 ± 1.36	18.57 ± 0.35	15.69 ± 0.62	19.83 ± 2.32

*Results are expressed as mean ± SD; each data point is the average of 2 repeated measurements from 3 independently replicated experiments

**The amount of cholesterol in the different fractions is expressed as a percentage of the initial concentration of cholesterol in the medium

***The amount of cholesterol not recovered is expressed as a percentage of the initial concentration of cholesterol in the medium

part (19.82%) was not recovered. Under these conditions, it was concluded that the removal of cholesterol from the broth might be attributed to coprecipitation of cholesterol with deconjugated bile salts (Klaver & Van Der Meer 1993), cell assimilation (Gilliland *et al*. 1985), and cholesterol degradation.

DISCUSSION

High cholesterol levels in blood are generally considered to be a risk factor for coronary heart disease. The effects of probiotic bacteria such as *L. acidophilus* on serum cholesterol levels have attracted much interest in recent years (Gilliland *et al*. 1985; Buck & Gilliland 1994; Ahn *et al*. 2003; Liong & Shah 2005; Park *et al*. 2007; Mathara *et al*. 2008). In the present study, we have shown that all the strains of the lactobacilli tested had the ability to remove cholesterol from laboratory media during growth. Among the strains tested, *L. plantarum* KLDS 1.0344 could remove as much cholesterol as 54.08% from the media. To administer the strain KLDS 1.0344 to humans, it will be necessary to assess its tolerance towards acid and bile, and its mechanism of cholesterol removal.

Probiotics are commonly delivered through food systems and must be acid and bile tolerant to survive in the human gastrointestinal tract. The time from entering the stomach to release is estimated to be approximately 90 min, with the following digestive processes requiring a longer residence time (Berrada *et al*. 1991). The microorganisms stresses begin in the stomach with its internal pH ranging between 1 and 3 (Azcarate-Peril *et al*. 2006). As shown above, a condition such as pH 2.5 for 2 h was selected to examine the acid tolerance of the tested strains. The strain *L. plantarum* KLDS 1.0344, with the highest acid tolerance, had more than 10^8 CFU/ml viable bacteria after 2 h of incubation at pH 2.5, and demonstrated a good tolerance towards acid. Oxgall, containing both conjugated and deconjugated bile salts, and similar in the composition of bile to that in the human body, was used as a bile source to study the bile tolerance of the tested strains. *L. plantarum* KLDS 1.0344 grew fastest in the presence of oxgall among the six tested strains. Therefore, *L. plantarum* KLDS 1.0344 might survive the low pH of the stomach environment and appear in the bowel system as active microbes.

The mechanism of cholesterol removal by *L. plantarum* KLDS 1.0344 for it to be effective as a probiotic was also investigated. There were three possible mechanisms underlying the ability of *L. plantarum* KLDS 1.0344 to remove cholesterol from the media. One was the coprecipitation of cholesterol with free bile salts. A part of cholesterol was precipitated and resolubilised in the washing fluid. This result was supported by the report of Klaver and Van Der Meer (1993), which states that cholesterol removal by Lactobacilli was due to bile salts deconjugation by BSH activity of the strains. Another mechanism was the assimilation of cholesterol by the cells of *L. plantarum* KLDS 1.0344. Cholesterol detected in fragmented-cells solution was entirely attributed to the assimilation of cholesterol by the cells of the strain (Gilliland *et al*. 1985; Grill *et al*. 2000). The third mechanism identified was the degradation of cholesterol by *L. plantarum* KLDS 1.0344, and this is reported here for the first time. In this study, cholesterol could not be entirely recovered from the supernatant fluid, washing fluid, and fragmented-cells solution. Partial intake of cholesterol into the cells of the strain must occur, and some cholesterol may be degraded into a nutritional ingredient used for

the growth of *L. plantarum* KLDS 1.0344. According to these results, further studies will follow on the exact course of cholesterol degradation by *L. plantarum* KLDS 1.0344.

In summary, *L. plantarum* KLDS 1.0344 strains showed stronger tolerance towards acid and bile, and a higher cholesterol removal capacity from media, indicating that it may be able to serve as a probiotic strain after further characterisation is completed by means of animal models and clinical tests.

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