

## Development technology of starter cultures using lactic acid bacteria isolated from fermented Camel milk with cholesterol lowering ability

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### ABSTRACT

The aim of the study is to develop a technology of starter cultures for fermented milk using new strains of lactic acid bacteria isolated from Mongolian traditional fermented camel milk. “*Khoormog*” samples are collected from Inner Mongolia, China. Totally 230 *Lactobacillus* strains are isolated and screened by acid-, bile- tolerance, lactose decomposition and acid production ability. The cholesterol lowering abilities and adhesiveness on Caco-2 are evaluated. The top 2 strains are identified as *Lactobacillus plantarum*. These 2 strains are prepared as the starter cultures in milk fermentation. The development technology of starter cultures is studied.

**Keywords:** Mongolian fermented Camel milk, Cholesterol lowering, *Lactobacillus plantarum*, starter cultures

### INTRODUCTION

The research of new functional foods that isolate and identify new strains of lactic acid bacteria from traditional dairy products may lead to novel bacterial strains with functional characteristics and revealing taxonomic characteristics.

It is known that camel has a long lactation period (more than 15 months). Camel milk is sticky milk with normal smell. It contains large contents of dry matter, high milk fat rate and small milk fat ball. As a good source of amino acids, camel milk is an ideal milk source. The most interesting characteristic is that the natural acidity of camel milk is at an average of 22 °T. It is 16-18 °T in cow milk and 6.5 °T in mares' milk. Camel milk can be preserved for a long time after processing or acidification without corruption, while cow milk cannot [1]. Traditional fermented camel milk, due to its rarity and high nutritional value, provides good conditions for microbial research [2, 3]. *Khoormog* is a unique Mongolian fermented camel milk with high nutritional value, unique flavor, and form many probiotic groups with good passage under the spontaneously fermentation [4]. As a rich resource of lactic acid bacteria, the fermented camel milk *Khoormog* was studied in the present study.

Current foods are rich in lipids. Cholesterol is an organic molecule, a type of lipid. Exogenous intake of cholesterol is mainly obtained from the diet. Thus, a higher intake from food leads to a net decrease in endogenous production and the increase of cholesterol in blood. Higher cholesterol in blood is associated with risks of heart disease [5-7]. Therefore, there has been considerable research on lowering cholesterol through a healthy and reasonable diet, especially through the use of lactic acid bacteria [8]. There are two theories support lactic acid bacteria to lower cholesterol. One theory is that some *in vitro* studies showed the cells of lactic acid bacteria strains can absorb cholesterol when grow in the high cholesterol medium containing bile salts under anaerobic conditions, and reduce the cholesterol in the medium [9]. The second theory derives from *in vitro* studies which showed the bile salt hydrolase (BSH) produced by lactic acid bacteria can deconjugate combined bile acid into free bile acid, the free bile acid combines with cholesterol into a complex to co-precipitate and lower the cholesterol [10, 11]. In this study, new strains of lactic acid bacteria are isolated from Mongolian traditional fermented camel milk in order to develop starter cultures with cholesterol lowering ability for fermented milk. The bacteria were

screened by acid-, bile-tolerance, lactose decomposition and acid production ability. The cholesterol lowering abilities and adhesiveness on Caco-2 were evaluated. The top 2 strains were prepared as the starter cultures for milk fermentation. The development of starter cultures and their fermentation characteristics are studied. The optimal fermentation temperature and optimal inoculum of development technology of starter cultures are studied by single factor experiments and response surface experiment design.

## EXPERIMENTAL

**Chemical compounds and viable counts:** Fermented camel milk *Khoormog* samples were originated from Inner Mongolia, China. A total number of thirteen *Khoormog* samples were collected in this study. The distribution of collected samples in Inner Mongolia was *Xilingol*, *Ulanqab*, *Ordos*, *Bayannur* and *Alxa* league cities. Samples were classified into three groups according to the collected regions (Table 1). The distribution of collected regions in Inner Mongolia, China was shown in Fig 1. Basic experiment was studied in Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolian Agricultural University in Hohhot, Inner Mongolia, China. The collected samples were stored in a refrigerator at 4 °C. After viable counts counting and chemical compounds analysis on the collected samples, it was frozen and stored.

The pH of each sample was directly measured using a pH meter (FE20, Mettler Toledo, China). The pH and titratable acidity (°T) were determined according to the Chinese national standard GB 5009. 239-2016 [12]. The lactose and organic acid content of *Khoormog* samples were determined by HPLC using the method reported by Kuda et al. [13]. The samples were centrifuged at 15,000 g for 3 min after diluting with nine volumes of distilled water.

Table 1. Collected location and group classification of *Khoormog* samples

Group No.	Number of collected samples	Sample ID	Location Group	Region
No. 1	N=4	T1		Sonid Right, Xilingol
		T2	IM_Midland	Siziwang, Ulanqab
		T3		Wushen, Ordos
		T4		Wushen, Ordos
No. 2	N=5	T5		Bayannur
		T6		Bayannur
		T7	IM_Midwest	Bayannur
		T8		Bayannur
		T9		Bayannur
T13	N=4	T10	IM_Western	Alxa Right
		T11		Alxa Right
		T12		Alxa Right
		T13		Alxa Left

The supernatant was passed through a 0.45 mm pore size filter and injected into the HPLC under the following conditions: column, IC Sep ICE-ORH-801, mobile phase 5 mMol/L of H<sub>2</sub>SO<sub>4</sub> with flow rate 0.8 mL/min, UV detector at 210 nm and column temperature 35 °C.

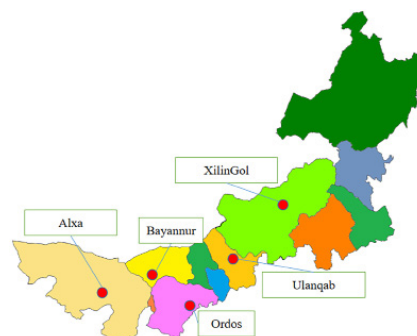


Fig. 1. Distribution of collected regions in Inner Mongolia, China

The viable count of lactic acid bacteria in each sample was counted according to the method of Kanno et al. [14] with some modification. 1 g of each sample and 9 mL of phosphate buffered saline solution (PBS, Biosharp, China, pH 7.2) containing 1 g/L agar were homogenized. Ten-fold serial dilutions (from 10<sup>-1</sup> to 10<sup>-5</sup>) of each sample were prepared. 50 µl of each dilution were directly inoculated on de Man Rogosa Sharpe plates (MRS agar, Oxoid, UK). The MRS plates were incubated at 37 °C and 25 °C under anaerobic conditions using the AnaeroPack system (Mitsubishi Gas Chemical, Japan) respectively. Viable count was counted and calculated after 48 h of incubation as log colony forming units (CFU) per gram of sample.

**Screening of acid- and bile- tolerance, lactose utilization and acid production ability:** Colonies grown on the MRS agar plates were isolated and purified after the viable count calculation. The yellow round shape colonies around with the clear zone were randomly selected. Ann average of 9-10 colonies were isolated from each sample incubated at two temperatures. A total of 230 colonies were isolated [13]. Gram positive, catalase negative and rod strains were isolated.

The lactic acid bacteria isolates were screened by acid-, bile- tolerance and lactose utilization tests by *in vitro*. The acid tolerance of each isolates were inoculated into 5 mL MRS broth which adjusted to pH 3.0. After 24 h incubation at 37°C, absorbance at OD<sub>600</sub> was measured, ΔA≥0.5 was chosen [15]. The bile tolerance of each isolate was determined by inoculation into 5 mL MRS broth containing 10 g/L bile (Oxgall, Neogen, USA). Absorbance at OD<sub>620</sub> was measured. A decrease in pH indicates the lactose utilization ability because of acidic fermentation products. Each isolate was inoculated into in Gifu Anaerobic Medium (GAM) semi-solid medium (Nissui, Japan) containing 1% (w/v) lactose. BTB-MR reagent (0.1 g bromothymol blue, 0.2 g methyl red, 300 mL ethanol, and 200 mL distilled water) was used [16].

According to acid-, bile- tolerance and lactose utilization tests, 34 lactic acid bacteria isolates were selected for acid production ability testing.

Acid production capacity was evaluated according to the method of Jicheng [17] with slight modification. Skimmed milk powder (Members Mark, New Zealand) was 10% (w/v) hydrated and heated at 95 °C for 5 min (pH 6.6) [18]. After cooling to 37 °C, lactic acid bacteria isolates were inoculated. The pH was recorded every 2 hours until the end of the fermentation (pH 4.6). The formation of milk curd was observed and a viable count undertaken. Based on sample origin, fermented milk observation and acid production ability, the top 9 lactic acid bacteria isolates were then selected for the subsequent experiments.

**16S rDNA gene sequence analysis:** The top 8 screened lactic acid bacteria isolates were identified using 16S rDNA gene sequence analysis. TIANamp bacteria DNA kit (TIANGEN, China) was used for the genomic DNA extraction. PCR amplification was performed according to the previous study by Jie [19]. Forward primer 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and reverse primer 1541R (5'- AAG GAG GTG ATC CAG CCG CA-3') was used [20]. PCR amplification program was as follow: 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and finally 72 °C for 5 min. DNA sequencing was performed by Shanghai Sangni Biotechnology Co., Ltd. The sequences were analyzed through the BLASTn database at GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

**Evaluation of cholesterol lowering ability by in vitro tests:** The evaluation of cholesterol lowering ability of the 8 isolates was studied in the College of Animal Science, Inner Mongolia Agricultural University, Inner Mongolia, China. The cholesterol lowering ability was assessed using a colorimetric assay [21]. MRS-THIO broth was supplemented with 0.3% (w/v) Oxgall as a bile salt. The sterilized cholesterol micelles (1 mg/mL in 0.4 M sucrose solution, provided by the Animal Research Laboratory of Inner Mongolia Agricultural University) was further added into the broth at a final concentration of 100 µg/mL. The 8 isolates were inoculated at 1 g/100 g and incubated under 37°C for 20 h. Uninoculated broth was used as control. Cells were centrifuged at 10,000g at 4 °C for 10 min. The total cholesterol content of the supernatant was measured. Mixed 2 mL of supernatant, 3 mL of 97% (w/v) ethanol (Sangon Biotech, China) and 2 mL of 50% (w/v) KOH (Feishuo, China). The mixture was heated at 60°C for 10 min, then cooled to room temperature. 5 mL of hexane was added, then vortexed and dried. o-phthalaldehyde (0.55 mg dissolved in 1 mL of acetic acid, Bomei, China) was added and incubated for 10 min. Absorbance at OD<sub>550</sub> was measured after adding 2 mL of H<sub>2</sub>SO<sub>4</sub> (Zhongzheng, China). The deconjugation of sodium taurocholate was assayed *in vitro*. MRS-THIO broth (OXOID, UK) was

supplemented with 0.2% (w/v) sodium taurocholate. The 8 isolates were inoculated at 1 g/100g into 20 mL MRS-THIO broth. Incubation under 37 °C for 24 h. Uninoculated broth was used as control. Deconjugation activity was determined by the content of free cholic acid using TBA Assay kit (Rui Chu, China) according to the method of Menghe [22]. MRS-THIO broth was supplemented with 0.2% (w/v) sodium taurocholate and 0.3% (w/v) oxgall. The sterilized cholesterol micelles were adjusted the same as above. The 8 isolates were inoculated at 1 g/100 g and incubated under 37°C for 20 h. The co-precipitation of cholesterol with cholic acid as liberated from the deconjugation of sodium taurocholate by the 8 isolates was measured as mentioned above.

The adhesiveness analysis of Caco-2 cells was measured according to the method of Menghe [22]. Caco-2 cells (HTB-37, American Type Culture Collection, USA) were incubated in DMEMF-12 culture. 20% (v/v) foetal bovine serum (FBS, Gibco, USA), 2% (v/v) antibiotics and anti-fungus solution (Sigma, China) was added into the DMEMF-12 culture. 24-well culture plates were used for the incubation of cells for two weeks, at 2.0×10<sup>5</sup> CFU/mL. Then washed twice using PBS solution (pH 7.4).

Single colonies of the isolates were pre-incubated under 37°C for 16 h. Cells were collected through centrifugation at 3000 g for 10 min. Washed twice using PBS solution. Adjusted the concentration to 5.0×10<sup>7</sup> CFU/mL [23]. The cells of the isolates were added into each well of the cultured Caco-2 cells. Incubation under 37°C for 1 h. For plate counting, trypsinase was added and the adhesiveness calculated. The evaluation of cholesterol lowering ability was measured in triplicate and compared with the standard *L. plantarum* strain P8.

#### **Preparation technology of stater cultures:**

The fermentation was studied in the Food Research Center of Mongolian University of Science and Technology, Mongolia. The 2 *L. plantarum* strains were prepared as the starter cultures in comparison with the standard *L. plantarum* strain P8. According to Li [23], the activated strains were pre-cultured in MRS broth for 16 h at 37 °C, then concentrated and freeze dried. Skim milk powder was 10% (w/v) hydrated and heat treated at 95 °C for 5 min and then cooled to 43 °C. The freeze dried strain powder was dissolved into the skim milk at 3% (w/v).

Then incubated at 43 °C, until it reached pH 4.6. Production starter culture was prepared after 3 times of activation. Production starter culture activity was inspected [24] and the acidity recorded every 2 hours of fermentation until pH 4.6. The initial acidity of skim milk was pH 6.7, titratable acidity 18 °T. The Resazurin reduction was tested [25], 1 mL of each production culture and 0.005% (v/v) of resazurin solution was dissolved into 9 mL skim milk. Incubation at 36.7 °C for 35 min.

**Determination of optimal temperature and inoculum in milk fermentation tests:**

The 2 *L. plantarum* strains were prepared as the starter cultures and fermented in cow's milk according to Gu [25] with some modification. Ultrahigh temperature (UHT) sterilized milk (whole milk, MLEKOVITA, Poland) was used. To determine the optimal temperature, the starter cultures of the 2 isolates were inoculated at 3 g/100 g of inoculum, and incubated at 37, 39, 41, 43 and 45°C. After 4 h of fermentation, the pH of each fermented milk was determined every 10 min until pH 4.6, then record the fermentation period. The viable count was counted. The water holding capacity of the fermented milk was calculated using the following method. Briefly, 20 g fermented milk (the solid part of the fermented milk without the extracted whey) was centrifuged at 5000 r/min for 15 min, rested for 10 min, weighed before and after removing the supernatant. The tastes were determined using the Taste Sensing System (SA402B, Insent, Japan). Briefly, 20 mL fermented milk was centrifuged at 5000 r/min for 10 min. Took 10 mL supernatant into the special cup of the Taste Sensing System using a pipette gun. Diluted with triply distilled water and stood for 1 h.

The tastes of sweetness, sourness, bitterness, astringency and umami were set and determined. Sensory evaluation was performed by seven trained researchers after milk fermentation according to Chinese standard RHB 103-2004 [26]. Briefly, the fermented milk samples were filled into 50 mL beakers and the color and texture was observed under the light. Researchers gargled with warm water after smelled the yogurt smell, then tasted. One hundred points system was used to evaluate the fermented milk. The evaluated sensory items included color (0-10 points), taste and smell (0-40 points), texture (0-50 points). The highest and the lowest points of evaluation were removed and the total points were calculated.

To determine the optimal inoculum, cow's milk was prepared as mentioned above.

The starter cultures of the 2 isolates were inoculated at 1, 3, 5, 8, and 10 g/100 g of inoculum respectively. The inoculated milk was incubated at 43°C. The acidity, viable count, water holding capacity, tastes and sensory evaluation was recorded.

All the above experiments were replicated three times in comparison with the standard *L. plantarum* strain P8. The analysis of variance (ANOVA) was performed using IBM SPSS statistics 25 programme software (Shown in Appendix). *Central composite design of optimal condition in milk fermentation:* According to the single factor experiment of determination of optimal temperature and inoculum, the optimal condition in milk fermentation of the 3 starter cultures was obtained through response surface experiment design. Central composite design was performed using the Design-Expert 12 software.

**RESULTS AND DISCUSSION**

**Chemical compounds and viable counts:** The chemical compounds and viable counts of the collected thirteen fermented camel milk *Khoormog* samples were studied. The acidity, chemical compounds and viable count of *Khoormog* are summarized in Table 2. The pH ranged from 3.4 to 5.0. Among the thirteen samples, three of the samples (T1, T2, and T3) collected from the group of the Midland of Inner Mongolia had high pH values, ranged from 4.8 to 5.0. The pH of samples in the other two groups ranged from 3.4 to 3.8. The organic acid contents of lactic acid of three samples (T1, T2, and T3) ranged from 329.3 to 789.7 mg/100 mL, acetic acid ranged from 21.6 to 57.3 mg/100mL. The organic acid contents of lactic acid of samples in the other two groups ranged from 632.3 to 1636 mg/100 mL, acetic acid ranged from 104.5 to 192.1 mg/100 mL. The viable count of samples ranged from 3.8 to 8.9 Log<sub>10</sub> CFU/g. Viable count of the samples in the group of the Midland of Inner Mongolia, ranged from 3.8 to 5.5 Log<sub>10</sub> CFU/g, in the other two groups ranged from 6.5 to 8.9 Log<sub>10</sub> CFU/g.

Table 2. Chemical compounds and viable count in the *Khoormog* samples in present study

Sample ID	pH	Titrate acidity (°T)	Lactose (g/100 g)	Lactic acid (mg/100 mL)	Acetic acid (mg/100 mL)	Viable count (Log <sub>10</sub> CFU <sup>*</sup> /g)
T1	5 ± 0.1*	150 ± 0	6.7 ± 0.1	329.3 ± 1.4	31.4 ± 0.1	5.5
T2	4.9 ± 0	158 ± 0	6.4 ± 0.1	539.5 ± 2	57.3 ± 0.3	3.8
T3	4.8 ± 0.1	153 ± 0	7.5 ± 0.1	789.7 ± 3.4	21.6 ± 0	5.5
T4	3.5 ± 0.1	153 ± 0	6.6 ± 0.1	1636 ± 2.6	166.4 ± 0.3	5.5
T5	3.4 ± 0.1	160 ± 1	6.2 ± 0.1	1608.3 ± 1.7	192.1 ± 0.4	8.4
T6	3.5 ± 0.1	154 ± 1	9 ± 0.1	1288.6 ± 0.3	169.4 ± 0.3	8.8
T7	3.5 ± 0.1	129 ± 0	7 ± 0.1	632.3 ± 1	159.3 ± 0.5	8.1
T8	3.4 ± .1	127 ± 1	6 ± 0.2	678.7 ± 2.4	104.5 ± 0.1	6.5
T9	3.5 ± 0.1	142 ± 1	4.8 ± 0.1	779.1 ± 2.3	112 ± 0.3	7.8
T10	3.8 ± 0.1	148 ± 1	6.7 ± 0.1	1537.5 ± 7.7	169.6 ± 0.7	7.6
T11	3.8 ± 0.1	123 ± 0	5.8 ± 0.1	1056.9 ± 1.2	134.4 ± 0.2	6.5
T12	3.6 ± 0.1	119 ± 0	5.9 ± 0.1	1281.5 ± 6.4	177.2 ± 0.5	8.9
T13	3.6 ± 0.1	123 ± 1	4.2 ± 0.1	826.2 ± 1.5	155.8 ± 0.8	7.5

Notice: \* Values are mean ± SD of triplicate measurement. CFU: colony forming unit.



In previous study by Shuangquan [29], the viable count of dairy products in Inner Mongolia ranged from 5.3 to 8.7 log CFU/g. Thus, three samples (T1, T2, and T3) collected from the group of the Midland of Inner Mongolia could be considered that it still in the early stage of fermentation. The samples in the other groups could be considered that it had entered late fermentation, and stored for a long time. Including the samples (T5 to T13) collected from the group of the Midwest and Western Inner Mongolia, showed high bacterial activities.

**Screening and identification:** Lactic acid bacteria were isolated from twenty three *Khoormog* samples according to their individual characteristics. Colonies grown on the MRS agar plates were purified and isolated. Among 230 gram positive and catalase negative rod strains, 172 grew in MRS broth adjusted to pH 3.0. Among the acid tolerance isolates, 69 produced acid from lactose.

Among the acid tolerance and lactose decomposed isolates, 34 grew in the 10 g/L bile condition. The 34 acid- and bile- tolerant and lactose decomposed isolates were tested by their acid production ability. Based on the sample origin, fermented milk observation and acid production ability, the top 8 isolates were selected. The acid production ability of the 8 isolates was shown in Table 3. The fermentation period ranged from 6.5 h to 7 h at the end of the fermentation (pH 4.6). The viable count ranged from 7.1 to 7.5 Log<sub>10</sub> CFU/g. The identification by 16S rDNA gene sequence analysis is shown in Table 4. The sequences were assembled and compared with the sequences available in GenBank through the BLASTn program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The other 8 strains were *L. plantarum* which isolated from Inner Mongolian, China. It belongs to *Bacteria; Terrabacteria group; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactiplantibacillus; Lactiplantibacillus plantarum* [30].

Table 3. Acid production ability of the 8 selected lactic acid bacteria isolates

Strains	Sample origin	Location group	Initial isolation temperature (°C)	Acidity	Fermentation period			Viable count (Log <sub>10</sub> CFU/g)
					4 h	6 h	8 h	
SQ9	T1	IM_Midland	25	pH °T*	5.7 ± 0* 31 ± 0	4.9 ± 0.1 61 ± 1	4.4 ± 0.1 92 ± 1	7.5
SQ10	T1	IM_Midland	37	pH °T	5.9 ± 0.1 27 ± 0	4.8 ± 0 65 ± 1	4.4 ± 0.1 90 ± 0	7.2
AQ1-7	T3	IM_Midland	37	pH °T	5.6 ± 0.2 35 ± 1	4.8 ± 0.1 65 ± 0	4.4 ± 0 89 ± 0	7.3
BM2-5	T6	IM_Midwest	37	pH °T	5.6 ± 0.1 35 ± 0	4.9 ± 0 60 ± 1	4.3 ± 0 94 ± 1	7.3
BM3-6	T7	IM_Midwest	37	pH °T	5.6 ± 0.1 35 ± 0	4.8 ± 0 67 ± 1	4.3 ± 0.1 93 ± 2	7.3
BM4-2	T8	IM_Midwest	25	pH °T	5.6 ± 0 34 ± 1	4.8 ± 0.1 66 ± 0	4.3 ± 0 94 ± 0	7.1
BM5-6	T9	IM_Midwest	37	pH °T	5.6 ± 0.1 34 ± 1	4.9 ± 0.1 63 ± 0	4.3 ± 0 94 ± 0	7.3
AM2-6	T11	IM_Western	25	pH °T	5.8 ± 0 30 ± 0	4.8 ± 0.1 68 ± 1	4.3 ± 0 94 ± 1	7.3

Notice: \* Values are mean ± SD of triplicate measurement. °T: Titratable acidity (°T).

Before any strains of functional bacteria can be used as a food adjunct, they must be able to survive in the acidic conditions in stomach and resist bile acids in order to be effective on the host [31]. Once ingested, the bacteria is exposed to the harsh digestive processes for more than 90 minutes before being released from the

stomach into the intestine [32]. Acid tolerance is often a crucial factor considering that pH of stomach could be as low as 2.5 in fasting subjects [19]. In addition, when probiotics pass through the upper intestinal tract, they are subjected to varying bile types and concentrations that may decrease their viability [31].

Table 4. Identification of the 8 lactic acid bacteria isolates by 16S rDNA gene sequence analysis

Strains	Description	Strain ID	Query cover	Query length	Identification percentage
SQ9	<i>L. plantarum</i>	WCFS1	100%	1440	100%
SQ10	<i>L. plantarum</i>	WCFS1	100%	1442	99.93%
AQ1-7	<i>L. plantarum</i>	WCFS1	100%	1440	100%
BM2-5	<i>L. plantarum</i>	WCFS1	100%	1438	100%
BM3-6	<i>L. plantarum</i>	WCFS1	100%	1441	99.93%
BM4-2	<i>L. plantarum</i>	WCFS1	100%	1439	99.93%
BM5-6	<i>L. plantarum</i>	WCFS1	100%	1442	100%
AM2-6	<i>L. plantarum</i>	WCFS1	100%	1439	99.93%

In order to simulate the human gastrointestinal environment, the acid- (pH 3.0) and bile- (10 g/L) tolerance tests were conducted *in vitro*. The lactose (1% w/v) utilization test was aimed at the lactose intolerance, which is mostly reported in Asia and Africa [13]. Lactose intolerance is a congenital hereditary disease in which the body does not produce lactase to utilize lactose. Flora decomposes lactose in the intestinal tract into lactic acid to destroy the alkaline environment of the intestine. Intestines have to secrete a large amount of alkaline digestive solution to neutralize the lactic acid which causes diarrhea [33]. Among the 230 lactic acid bacteria isolates, 34 acid- and bile- tolerance and lactose decomposed isolates were screened and their acid production ability was tested. Based on the sample origin, fermented milk observation and acid production ability, the top 8 isolates were selected for the further functional experiment.

**Evaluation of cholesterol lowering ability by *in vitro* tests:** The cholesterol lowering ability of the 8 isolates ranged from 10.08±0.13 µg/mL to 48.56±4.33 µg/mL. The cholesterol lowering rate ranged from 8.94 to 32.37% (Table 5). It matched the previous study by Shehata [34] that the cholesterol lowering ability ranged from 11.97 to 77.61 µg/mL, and the cholesterol lowering rate ranged from 7.98% to 51.74%. Strain AM2-6 showed a high cholesterol lowering ability that removed cholesterol 48.56±4.33 µg/mL, at a rate of 32.37%. Strain BM2-5 was followed after strain AM2-6, removed cholesterol 43.47±2.93 µg/mL, at a rate of 28.98%. Strain BM4-2 removed cholesterol 39.71±5.61 µg/mL, at a rate of 26.47%. The standard strain P8 removed cholesterol 17.65±2.12 µg/mL, at a rate of 11.76%. These results demonstrate that the cells of 8 isolates could absorb cholesterol when grow in the high cholesterol medium containing bile salts under anaerobic conditions, and function *in vivo* to exert a hypocholesterolemic effect [22]. Based on the cholesterol lowering ability, the *L. plantarum* strains AM2-6, BM2-5 and BM4-2 were selected as the pending strains for further fermentation experiment. Deconjugation of sodium taurocholate of the 8 isolates

ranged from 1.12±0.07 µM/mL to 2.04±0.21 µM/mL (Table 5). It could be considered that the 8 isolates could produce BSH, the enzyme acting on bile salts deconjugation, to deconjugate bile salts in agreement with the previous study by Shehata [34]. The *L. plantarum* strain AM2-6 showed a high BSH activity. It released 2.04±0.21 µM/mL of cholic acid, and significantly higher than the other 7 isolates (P<0.05). It was followed by *L. plantarum* strains BM2-5 and BM4-2, the cholic acid released 1.61±0.14 µM/mL and 1.60±0.27 µM/mL respectively. The *L. plantarum* strain P8 released 1.13±0.05 µM/mL cholic acid. As a known comparative strain, strain P8 had a certain BSH activity, and it was similar with the other 5 isolates (SQ9, SQ10, AQ1-7, BM3-6, BM5-6, P>0.05). The coprecipitation of cholesterol with cholic acid as liberated from the deconjugation of sodium taurocholate by the 8 isolates ranged from 1.06±0.11 µg/mL to 1.4±0.04 µg/mL (Table 5, p>0.05). At concentrations similar to human bile, more than 1.4±0.04 µg/mL of cholesterol was the precipitated from the deconjugation activity by the *L. plantarum* strain AM2-6. It was followed by *L. plantarum* strains BM2-5 and BM4-2, precipitated 1.38±0.06 µg/mL and 1.33 ± 0.07 µg/mL of cholesterol from the deconjugation activity. *L. plantarum* strains AM2-6, BM2-5 and BM4-2 were selected as the pending strains for further fermentation experiment. The decrease of cholesterol level is directly related to the intestinal adhesion of lactic acid bacteria in dairy products. The adhesiveness on Caco-2 cells of the 8 isolates ranged from 0.76±0.21 to 1.84±0.02 (Table 5). Strain BM2-5 showed a high adhesiveness on Caco-2 cells at 1.84±0.02. Strain AM2-6 was followed at 1.69±0.06. Strain AQ1-7 was 1.58±0.02. The standard strain P8 was 1.19±0.01. Therefore, the *L. plantarum* strains AM2-6 and BM2-5 could be used as probiotics in functional foods to reduce serum cholesterol. Cholesterol lowering ability of the 8 isolates was evaluated by *in vitro* tests of cholesterol lowering ability and deconjugation of sodium taurocholate. The 8 isolates showed a certain degree of cholesterol lowering ability and deconjugation of sodium taurocholate.

Table 5. Cholesterol lowering ability, BSH activity on sodium taurocholate, cholesterol precipitation with deconjugation of sodium taurocholate and Caco-2 Cell adhesiveness of the 8 isolates

Strains	Lowering cholesterol (µg/mL)	Cholesterol lowering rate (%)	BSH activity on sodium taurocholate (µM/mL)	Precipitated cholesterol by deconjugation of sodium taurocholate (µg/mL)	Caco-2 cell adhesiveness
SQ9	18.3 ± 1.39	12.20	1.22 ± 0	1.32 ± 0	1.4 ± 0.1
SQ10	15.92 ± 3.43	10.61	1.12 ± 0.07	1.19 ± 0.09	1.09 ± 0.04
AQ1-7	10.08 ± 0.13	6.72	1.16 ± 0	1.14 ± 0	1.58 ± 0.02
BM2-5	43.47 ± 2.93	28.98	1.61 ± 0.14	1.38 ± 0.06	1.84 ± 0.02
BM3-6	13.41 ± 2.26	8.94	1.24 ± 0.14	1.27 ± 0.03	0.76 ± 0.21
BM4-2	39.71 ± 5.61	26.47	1.6 ± 0.27	1.33 ± 0.07	1.36 ± 0
BM5-6	24.71 ± 6.7	16.47	1.18 ± 0.14	1.16 ± 0.02	1.1 ± 0
AM2-6	48.56 ± 4.33	32.37	2.04 ± 0.21	1.4 ± 0.04	1.69 ± 0.08
STD*	17.65 ± 2.12	11.76	1.13 ± 0.05	1.06 ± 0.11	1.19 ± 0.01

Notice: \* Values are mean ± SD of triplicate measurement. STD: *L. plantarum* P8

The top two *L. plantarum* isolates were AM2-6 and BM2-5. At concentrations similar to human bile, strain AM2-6 removed cholesterol  $48.56 \pm 4.33 \mu\text{g/mL}$ , at a rate of 32.37%, and released  $2.04 \pm 0.21 \mu\text{M/mL}$  of cholic acid, more than  $1.4 \pm 0.04 \mu\text{g/mL}$  of cholesterol was precipitated from the deconjugation activity. Strain BM2-5 removed cholesterol  $43.47 \pm 2.93 \mu\text{g/mL}$ , at a rate of 28.98% and released  $1.61 \pm 0.14 \mu\text{M/mL}$  cholic acid, precipitated  $1.38 \pm 0.06 \mu\text{g/mL}$  of cholesterol. As a known comparative strain, P8 removed cholesterol  $17.65 \pm 2.12 \mu\text{g/mL}$ , at a rate of 11.76%, released  $1.13 \pm 0.05 \mu\text{M/mL}$  cholic acid, precipitated  $1.06 \pm 0.11 \mu\text{g/mL}$  of cholesterol from the deconjugation activity. In the previous study by Kuda *et al.* [35], the cholesterol lowering rate of the eleven *L. plantarum* strains isolated from Japanese surfperch fish were over 60%. In the previous study by Simona B [36], two of the eight *L. plantarum* strains isolated from Italian dairy product had cholesterol lowering ability. The cholesterol lowering rate was 18.4% and 20.5% respectively. In the previous study by Mahdieh [37], ten lactic acid bacteria isolated from Iranian dairy product showed cholesterol lowering ability, however no *L. plantarum* strains were screened. The cholesterol lowering rate was ranged from 28% to 83%. In the previous study by Menghe [22], three *L. helveticus* strains were isolated from Mongolian dairy product showed cholesterol lowering ability at the rate of 7.98%, 41.03% and 51.74% respectively. The *L. plantarum* strains didn't show any cholesterol lowering ability. Combined with the previous studies, the specific lactic acid bacteria strains showed certain degrees of cholesterol lowering ability. It is neither limited to the species of lactic acid bacteria nor the isolation sample origin. Compared to the strains isolated from dairy products in the previous study, the *L. plantarum* strains AM2-6 and BM2-5 in the present study, showed relatively high cholesterol lowering ability. However, the *L. plantarum* strains isolated from Japanese surfperch fish showed higher cholesterol lowering abilities. It is considered important to adhesive to the intestinal mucous membrane. Caco-2 cells are the human intestinal cell lines to describe normal human enterocytes [22]. The 8 isolates showed certain degree of adhesiveness on Caco-2 cells. BM2-5 showed a high adhesiveness on Caco-2 cells at  $1.84 \pm 0.02$ . AM2-6 was followed at  $1.69 \pm 0.08$ . P8 was  $1.19 \pm 0.01$ . According to the cholesterol lowering abilities and adhesiveness on Caco-2 cells, the

*L. plantarum* strains AM2-6 and BM2-5, were selected as the typical strains for the preparation technology of starter cultures in comparison with the standard *L. plantarum* strain P8.

**Preparation technology of stater cultures:** The 2 *L. plantarum* strains AM2-6 and BM2-5 were selected as the typical strains to use as the starter cultures in comparison with the standard *L. plantarum* strain P8. Production starter culture of AM2-6, BM2-5 and P8 was prepared as the flow chart in Fig 2.

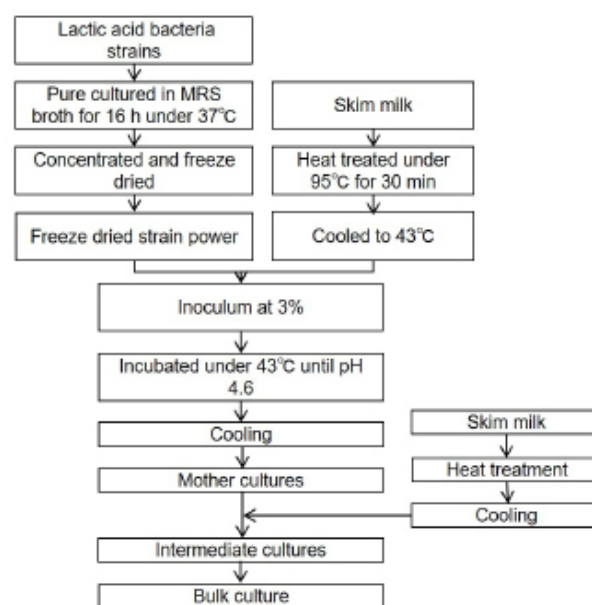


Fig. 2. The flow chart of preparation process of starter cultures

Production starter culture activity of AM2-6, BM2-5 and P8 was recorded (Table 6). The starter cultures of AM2-6, BM2-5 and P8 showed an acid production ability during 8 h fermentation. The fermentation period of starter cultures of AM2-6, BM2-5 and P8 were about 7 h (pH 4.6). Starter cultures of AM2-6 and BM2-5 showed a higher acid production ability than the known P8 during the 4 h to 6 h of fermentation. Among them, AM2-6 showed stronger acid production ability in a short time ( $p < 0.05$ ). This is consistent with the previous studies by Pan *et al.* [38]. In a previous study by Song [39], the fermentation period of *L. plantarum* starter cultures was about 8 h to 9.3 h. Resazurin solution of the 3 starter cultures were completely faded indicating

Table 6. Production starter culture activity of *L. plantarum* AM2-6, BM2-5 and P8, recorded by acidity

No.	Strains	Fermentation period							
		2 h		4 h		6 h		8 h	
Acidity		pH	°T*	pH	°T	pH	°T	pH	°T
No.4	BM2-5	$6.1 \pm 0^*$	$24 \pm 0$	$5.6 \pm 0.1$	$36 \pm 0$	$4.8 \pm 0$	$64 \pm 0$	$4.4 \pm 0$	$91 \pm 0$
No.8	AM2-6	$6.0 \pm 0.2$	$24 \pm 0$	$5.4 \pm 0.1$	$41 \pm 0$	$4.7 \pm 0.1$	$67 \pm 1$	$4.3 \pm 0.1$	$94 \pm 2$
No.9	STD*	$6.2 \pm 0$	$21 \pm 0$	$5.8 \pm 0$	$30 \pm 0$	$4.9 \pm 0$	$61 \pm 1$	$4.4 \pm 0.1$	$92 \pm 1$

Notice: \* Values are mean  $\pm$  SD of triplicate measurement. STD: *L. plantarum* P8. °T: Titratable acidity (°T)

that the 3 of *L. plantarum* starter cultures kept high activity and could be used in the milk fermentation.

**Determination of optimal temperature in milk fermentation tests:** *L. plantarum* starter cultures of AM2-6, BM2-5 and P8 were provided for cow's milk fermentation. To determination of optimal fermentation temperature, 37, 39, 41, 43, and 45°C were trialed. The inoculum used was 3 g/100 g milk. A pH of 4.6 was taken to be the end of fermentation. The optimal fermentation temperature for the fermented milk should meet the following conditions. Viable count should over 6 Log<sub>10</sub> CFU/g [40]. The curd texture is moderate with milky white color, with no whey separated. Keeping high water holding capacity, good tastes and high sensory score are also important.

The taste determination by Taste Sensing System of the fermented milk is shown in Fig 3. The tastes were set as sweetness, sourness, bitterness, astringency and umami. The tastes of sourness, bitterness and umami showed significant differences ( $p < 0.05$ ), sweetness and astringency didn't show significant differences ( $p > 0.05$ ). It indicated that the fermentation temperature had an effect on the taste of the fermented milk. The taste of sourness showed the most significant differences in the three fermented milk, it was followed by bitterness and umami.

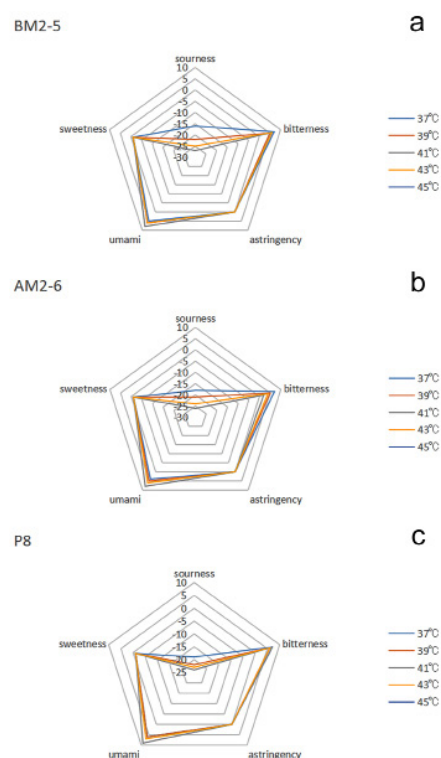


Fig. 3. Tastes determination by Taste Sensing System in different fermentation temperature. (a) BM2-5, (b) AM2-6, (c) P8

Table 7. Fermentation period, viable count, water holding capacity and sensory evaluation of the fermented milk produced by *L. plantarum* starter cultures of AM2-6, BM2-5 and P8 in different fermentation temperature (pH 4.6)

Starter culture	Fermentation temperature, °C	Fermentation period	Viable count (Log <sub>10</sub> CFU/g)	Water holding capacity (%)	Fermented milk state	Sensory score
BM2-5	37	7 h 50 min	6.7	59.8 ± 0*	The curd texture is soft, some whey separated. Milky white color.	70
	39	7 h 50 min	6.8	61.2 ± 0.1	The curd texture is slightly soft, some whey separated. Milky white color.	74
	41	7 h 30 min	6.9	62.4 ± 0.1	The curd texture is slightly soft, some whey separated. Milky white color.	89
	43	7 h 10 min	7.0	65.7 ± 0.1	The curd texture is moderate, no whey separated. Milky white color.	90
	45	7 h 10 min	6.8	63.5 ± 0.1	The curd texture is slightly soft, no whey separated. Milky white color.	87
AM2-6	37	7 h 40 min	6.5	60.1±0.2	The curd texture is soft, some whey separated. Milky white color.	71
	39	7 h 40 min	6.7	61.9±0.3	The curd texture is soft, some whey separated. Milky white color.	72
	41	7 h 20 min	7.1	64.7 ± 0.1	The curd texture is moderate, no whey separated. Milky white color.	87
	43	7 h	7.3	66.2 ± 0.3	The curd texture is moderate, no whey separated. Milky white color.	91
P8	37	7 h	7.1	64.9 ± 0.1	The curd texture is slightly soft, no whey separated. Milky white color.	88
	37	7 h 50 min	6.4	57.4 ± 0	The curd texture is soft, some whey separated. Milky white color.	68
	39	7 h 40 min	6.6	58.6 ± 0.1	The curd texture is slightly soft, little whey separated. Milky white color.	72
	41	7 h 20 min	6.7	59.7 ± 0.3	The curd texture is slightly soft, no whey separated. Milky white color.	87
	43	7 h 20 min	6.9	61.6 ± 0	The curd texture is moderate, no whey separated. Milky white color.	90
STD*	43	7 h 20 min	6.9	61.6 ± 0	The curd texture is moderate, no whey separated. Milky white color.	90
	45	7 h 20 min	6.9	59.5 ± 0.1	The curd texture is slightly soft, no whey separated. Milky white color.	88

Notice: \* Values are mean ± SD of triplicate measurement. STD: *L. plantarum* P8



In general, the sourness increased as the fermentation temperature increased whilst bitterness and umami decreased as the fermentation temperature increased. It indicated that the increase in sourness can not only reduce bitterness in the fermented milk, but also reduce umami taste. The fermentation period, viable count, water holding capacity and sensory evaluation of the fermented milk in different fermentation temperature is shown in Table 7.

The fermentation periods of the three fermented milk decreased as the fermentation temperature increased. According to the viable count, water holding capacity and sensory evaluation of the fermented milk, the optimal temperature for the fermentation of milk was 43°C. At 43°C the fermentation period of the 3 starter cultures was about 7 to 7.5 h. In the case of AM2-6, viable count of the fermented milk was 7.3 Log 10 CFU/g, fermentation period was 7 h. The curd texture was moderate, milky white color, and no whey separated. Water holding capacity was 66.2%. Sensory score was 91.

In the case of BM2-5, viable count was 7.0 Log10 CFU/g, fermentation period was 7 h 10 min, water holding capacity was 65.7% and sensory score was 90. In the case of P8, viable count was 6.9 Log 10 CFU/g, fermentation period was 7 h 20 min, water holding capacity was 61.6%, sensory score was 90.

In major dairy factories, the fermentation temperature of the fermented milk ranges from 37 to 43°C [41]. In other words, the differences of the fermentation temperature depend on the differences of the starter cultures. Therefore, the optimal fermentation temperature of the two *L. plantarum* strains AM2-6 and BM2-5 were determined for a better understanding of the potential health benefits. The fermentation temperatures trialed in the present study were 37, 39, 41, 43 and 45°C. According to the previous study by Hao [42], both high and low temperature fermentation reduced the water holding capacity of the fermented milk, a fermentation temperature between 36°C and 44°C had no significant effects on the water holding capacity of fermented milk. In the present study, the water holding capacity of fermented milk showed significant differences during fermentation between 37°C and 45°C ( $p < 0.05$ ). Both AM2-6 and BM2-5 showed a high water holding capacity under 43°C of fermentation, and significantly higher than the known P8 ( $p < 0.05$ ). In general, the fermentation periods decreased as the temperature increased. Viable count was over 6 Log 10 CFU/g in all of the fermented milk. Combined with the tastes determination by Taste Sensing System and sensory evaluation by seven trained researchers, the fermentation temperature has certain effects on the fermented milk. The taste of sourness increased, the bitterness and umami decreased as the fermentation temperature increased. The differences of sourness supposed to related to the growth temperature of *L. plantarum* strains.

The differences of bitterness and umami may also have related to free amino acids in the fermented milk. For example, glutamic acid (Glu) has an umami taste and glycine (Gly) has a sweet taste [25]. The fermented milk produced under 43°C by both AM2-6 and BM2-5 showed a good taste.

#### Determination of optimal inoculum in milk fermentation tests:

To determine the optimal inoculum of the starter cultures 1, 3, 5, 8, and 10 g/100 g of inoculum respectively. The inoculated milk was incubated at 43°C with pH 4.6 indicating the end of the fermentation. The optimal inoculum of the fermented milk should meet the conditions as mentioned above.

The taste determination in different inoculum is shown in Fig 4.

The tastes of sourness, bitterness and umami showed significant differences ( $p < 0.05$ ), sweetness and astringency didn't show significant differences ( $p > 0.05$ ). The fermentation period, viable count, water holding capacity and sensory evaluation of the fermented milk in different inoculum is shown in Table 8. When increased or decreased the inoculum of the starter culture, the fermentation period was prolonged, and sensory evaluation was decreased.

Combined the viable count, water holding capacity and sensory evaluation of the fermented milk shown in Table 8, the optimal inoculum of the starter cultures AM2-6 and BM2-5 was 3 g/100 g, and P8 was 5 g/100 g.

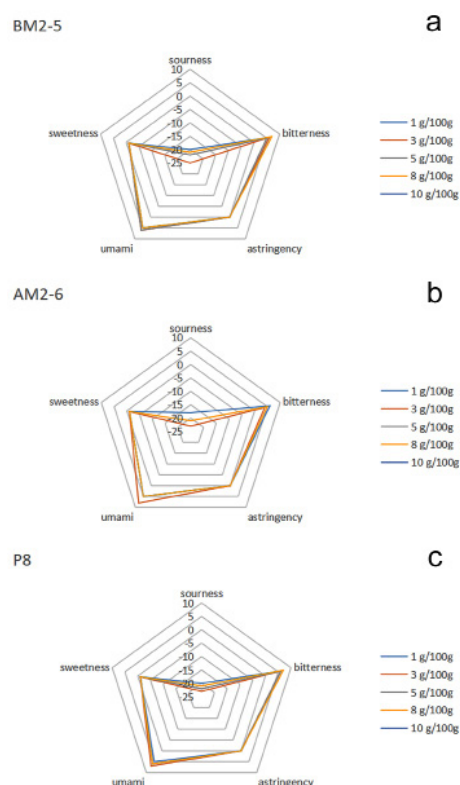


Fig. 4. Tastes determination by Taste Sensing System in different inoculum. (a) BM2-5, (b) AM2-6, (c) P8

Table 7. Fermentation period, viable count, water holding capacity and sensory evaluation of the fermented milk produced by *L. plantarum* starter cultures of AM2-6, BM2-5 and P8 in different inoculum (pH 4.6)

Starter culture	Inoculum g/100g	Fermentation Period	Viable count (Log 10 CFU/g)	Water holding capacity (%)	Fermented milk state	Sensory score
BM2-5	1	7 h 50 min	6.5	60.3 ± 0.1*	The curd texture is soft, some whey separated. Milky white color.	72
	3	7 h 10 min	7.0	65.7 ± 0.3	The curd texture is moderate, no whey separated. Milky white color.	89
	5	7 h 30 min	6.7	63.4 ± 0	The curd texture is slightly soft, no whey separated. Milky white color.	86
	8	7 h 40 min	6.3	61.8 ± 0.2	The curd texture is slightly soft, some whey separated. Milky white color.	78
	10	7 h 50 min	6.2	60.7 ± 0.2	The curd texture is slightly soft, some whey separated. Milky white color.	74
AM2-6	1	7 h 40 min	6.6	61.3 ± 0.3	The curd texture is soft, some whey separated. Milky white color.	70
	3	7 h	7.3	66.2 ± 0.2	The curd texture is moderate, no whey separated. Milky white color.	90
	5	7 h	7.1	63.0 ± 0.2	The curd texture is slightly soft, no whey separated. Milky white color.	88
	8	7 h 20 min	6.7	61.2 ± 0.2	The curd texture is slightly soft, some whey separated. Milky white color.	77
	10	7 h 40 min	6.4	58.6 ± 0.2	The curd texture is slightly soft, some whey separated. Milky white color.	75
STD*	1	7 h 50 min	6.5	54.3 ± 0.2	The curd texture is soft, some whey separated. Milky white color.	78
	3	7 h 40 min	6.7	59.9 ± 0	The curd texture is slightly soft, no whey separated. Milky white color.	86
	5	7 h 20 min	6.9	62.3 ± 0	The curd texture is moderate, no whey separated. Milky white color.	88
	8	7 h 40 min	6.8	60.7 ± 0.1	The curd texture is slightly soft, some whey separated. Milky white color.	84
	10	7 h 50 min	6.5	56.5 ± 0.2	The curd texture is slightly soft, some whey separated. Milky white color.	77

Notice: \* Values are mean±SD of triplicate measurement. STD: *L. plantarum* P8

**Central composite design of optimal condition in milk fermentation:** In central composite design, two numeric factors and three responses were set. The two numeric factors were fermentation temperature and the dose of inoculum in each fermented milk, the three responses were fermentation period, viable count and water holding capacity.

The desirable optimal condition in milk fermentation according to the central composite design is shown in Fig 5. In the case of AM2-6, the optimal fermentation temperature is 43.9°C, the optimal inoculum is 3.9 g/100 g. In the case of BM2-5, the optimal fermentation temperature is 43.3°C, the optimal inoculum is 3.6 g/100 g. In the case of P8, the optimal fermentation temperature is 43.6°C, the optimal inoculum is 5.4 g/100 g.

The results of response surface design by central composite design were consistent with the single factor experiments.

Therefore, combined with the results of single factor experiment and response surface design, the optimal fermentation conditions of the *L. plantarum* starter cultures AM2-6, BM2-5 and P8 were as follows. The optimal fermentation temperature was 43°C for the three *L. plantarum* starter cultures. The optimal inoculum of AM2-6 and BM2-5 was 3 g/100 g, and P8 was 5 g/100 g.

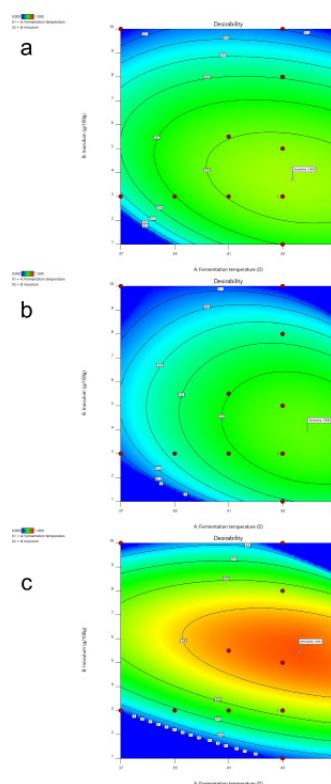


Fig. 5. Desirable optimal condition in milk fermentation according to the central composite design. (a) BM2-5, (b) AM2-6, (c) P8

## CONCLUSIONS

In this study, thirteen fermented camel milk *Khoormog* samples were collected from cities across Inner Mongolia, China. According to the analyses, the *Khoormog* samples provided high nutritional values and microbial conditions for research. A total number of 230 lactic acid bacteria strains were isolated and screened using acid-, bile- tolerance, lactose decomposition and acid production ability. Based on the sample origin, fermented milk observation and acid production ability, the top 8 *L. plantarum* isolates were selected for the further functional experiment. The cholesterol lowering abilities and adhesiveness on Caco-2 are evaluated. All the strains showed a certain degree of cholesterol lowering ability. The results demonstrated that the *L. plantarum* strains AM2-6 and BM2-5 could produce BSH to deconjugate bile salts in order to reduce cholesterol. And also, the cells of *L. plantarum* strains AM2-6 and BM2-5 could absorb cholesterol when grow in the high cholesterol medium containing bile salts under anaerobic conditions, and function *in vivo* to exert a hypocholesterolemic effect. The *L. plantarum* strains AM2-6 and BM2-5 showed high adhesiveness on Caco-2 cells. The decrease of cholesterol level is directly related to the intestinal adhesion of lactic acid bacteria in dairy products. Therefore, the *L. plantarum* strains AM2-6 and BM2-5 could be used as *probiotics* in functional foods to reduce serum cholesterol. These two strains were selected as the typical strains for the preparation technology of starter cultures in comparison with the known *L. plantarum* strain P8 in milk fermentation. The optimal fermentation temperature and optimal inoculum of development technology of starter cultures were studied by single factor experiments and response surface experiment design. As mentioned above, not every *L. plantarum* strains have the ability to ferment in milk. The resource of isolation is important. In this study, AM2-6 and BM2-5 could be used for the fermented milk production with high fermentation ability. Even compared to the known P8, AM2-6 could exceed P8 in cow's milk fermentation.

## ACKNOWLEDGMENT

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