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Cholesterol-Lowering Potentials of Lactic Acid Bacteria with potential Probiotic Properties

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Abstract: Probiotic organisms, lactic acid bacteria (LAB), commonly used in fermented dairy products. In this study, selected strains of LAB were isolated from fermented food or probiotic products. After screening for the relevant properties of probiotic organisms, five promising isolates were identified as *Pediococcusplantarum*L14/1, *P. acidilactici* L25,*Lactobacillus plantarum*L26, *Lb. pentosus*, *Enterococcus faecium* N 15 which were tested for capability to remove cholesterolfrom the culture medium. These finding showed that a considerable variation existed among cultures in their growth viability in the presence of bile saltand assimilation of cholesterol from the medium. All tested strains removed less cholesterol from the broth compared to those grown in broth supplemented with 0.3% bile salts (oxgall), especially L26 and N15. *Lb. plantarum* L26 appeared to be more active compared to the other strains, and therefore, is regarded as a suitable candidate probiotic and adjunct culture.
Keywords: Antimicrobial activity;Cholesterol Removal; Lactobacillus;Probiotics.

Introduction

Cholesterol is a vital substance in the human body. Long standing elevated levels of blood cholesterol may lead to atherosclerosis and may therefore pose a major risk for developing cardiovascular diseases (CVDs) and colon cancer[1]. The drug therapy and nonpharmacologic approaches, including dietary intervention, behaviour modification, and regular exercise, are common strategies to lower blood cholesterol levels[2]. Despite the proven cholesterol-lowering ability of certain pharmacological agents, unwanted side effects can occur in some cases, such as gastrointestinal discomfort[3].Interestingly, these cholesterol levels could be brought down using probiotic bacteria[4].

Probiotic bacteria have multiple potential health effects, including blocking gastroenteric pathogens[5-8], neutralizing food mutagens produced in the colon[5,9-13], enhancing the immune response[10,12,14-17], lowering serum cholesterol, and stopping intestinal dysfunction[18-24]. Recently, lactic acid bacteria (LAB)known as probiotic bacteria have attracted attention as potential cholesterol-lowering agent[25]. Consumption of dairy products containing probiotics has been proposed as a means to lower serum cholesterol[26]. Several studies indicated consumption of certain cultured dairy products could lower total plasma cholesterol and low-density lipoprotein cholesterol[1,27]. The fermented milk containing a wild *Lactobacillus* strain was reported to have a hypocholesterolemic(cholesterol-lowering) effect in humans[28]. Since then, many experiments have been conducted *in vitro* or *in vivo* to investigate the hypocholesterolemic effect of LAB, especially strains of *Lactobacillus* and *Bifidobacterium*[29-31]. In a review, they concluded that dairy products fermented with the appropriate strain(s) of bacteria might induce a decrease in the level of circulating cholesterol concentrations[32]. However, the strains found in fermenteddairy products do not normally reside in the humanintestinal tract. Thus, daily consumption of probioticproducts may be a dietary solution for inducing long-termhypocholesterolemic effects.

In general, probiotic bacteriamust colonize the gastrointestinal tract (GIT) of the host, have acid- and bile salt-tolerance, and block putrefactive bacteria in the GIT. They have been used in fermented foods for several centuries without adverse effects[33,34] and are classified as Generally Recognized as Safe (GRAS) because of their long history of safe use, particularly in dairy foods[35,36]. Here, we evaluated the ability of strains of LAB isolated from fermented food to *in vitro* as a cholesterol-reducing probiotic.

Materials and Methods

Bacterial strains

The origins of the strains used in this study are shown in Table 1[37]. Isolation of LAB strains was performed from fermented foodsor probiotic products, collected from the retrail market of southern Thailand. These samples were serially diluted 10-fold from10^{-1} to 10^{-8}, and 100 µl was spread onto MRS agar. After 48 h of incubation in microaerobicallyconditions (5%CO_2)at 37°C, white opaque colonies 2-3mmin diameterwere randomly selected and purified. Only Gram-positive and catalase-negative strains were takenas presumptive LAB and stored at 4 °C in MRS agar plate for further study. The strains were tested for their probiotic characteristic *i.e.* bile salt tolerance, antibacterial activity. An MRS broth containing 15% glycerol was used to preserve the cultures at -80°C.

Table 1 List of bacterial strain used in this study

Bacterial strains	Source	Origin
Pediococcus pentosaceus 1 L14/1	Isolate[a]	Fermented food
P. acidilactici L25	Isolate	Fermented food
Lactobacillus plantarum L26	Isolate	Fermented food
Lb. pentosus	Isolate	Probiotic products
Enterococcus faecium N 15	-	(38)

[a]Isolated from fermented foods or probiotic products

Identification of LAB by API 50 CHL assay

For primary species-specific identification, bacterial strains isolated from fermented foods of probiotic products were subjected to API 50 CHL (bioMerieux, France) assay. Purified strains were cultivated on MRS plates in anaerobic vessels. Grown colonies were cultivated in 10 ml of MRS medium broth at 30°Cfor 48 h., after that the culture was washed and resuspended into 0.85% NaCl. The turbidity of the suspension was determined by the McFarland method according to the instructions provided by the manufacturer. Cell suspension was applied into API 50 CH strip wells, which were coated with paraffin oil. The strips were incubated at 30 °C. The result was read after 24 h and verified after 48 h. Fermentation of carbohydrates in the carbohydrate medium was indicated by a yellow colour except for esculine (dark brown). Colour reactions were scored against a chart provided by the manufacturer.

Microbial Inocula

The microbial inocula strains, *ie.*,*Staphylococcus aureus* TISTR 517, *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781, and fungus *Candida albicans* DMST 5239, were used as indicating strains in this study. The bacterial inocula strains or fungus *C. albicans* were grown on TGE (tryptone-glucose-yeast extract) or SD-agar plate (Sabouraud Dextrose), respectively. The bacterial inocula were grown at 37°C for 1 day, while the *C. albicans* was grown at 30°C for 2 days and then stored at 4°C. Microbial cells inocula were prepared from washed cultures grown in TGE or SD broth (Oxoid) and still cultured at 30°C for 1 day. Microbial cell concentrations were determined using Mcfarland No.0.5, and adjusted to 10^6 per ml of sterile peptone water (2%)

Antimicrobial activity assay

The cross streak method was used to detect antimicrobial activity. All experiments performed in duplicate. The cross streak method was performed using TGE[39] agar plates on which selected isolates were inoculated as 7.5-cm long lines, 0.6-cm in width, and incubated at 37°C for 3 days in 5% CO_2 atmosphere. The plates were then cross streaked with microbial inocula strains, incubated aerobically at 37°C for 24 h. The plates were examined to inhibit growth of microbial inocula strains around the streak line of selected isolates.

Acid tolerance

Acid tolerance of the cultures was investigated by incubating the organisms in MRS broth. The pH was adjusted to 2.5 with HCl and cultures were incubated at 37 °C for 3 h. Each of the isolated LAB was subcultured at least 3 times before experimental use, followed by centrifugation after the final subculture, inoculation (10% v/v) into the broth to concentration of 10^7 cfu/ml, and growth monitoring using the plate count method[40]. The experiments were repeated twice.

Bile salt tolerance

The LAB isolates were analyzed for their resistance to bile salt. The MRS broths at concentrations of 0.3% (w/v) of oxgall were prepared and dispensed in 10 mLvolumes and sterilized by heating 121 °C for 15 min. Each of the isolated LAB was subcultured at least 3 times before experimental use, followed by centrifugation after the finalsubculture, inoculation (10% v/v) into the broth to concentration of 10^7 cfu/ml , and growth monitoring using the plate count method[(40)]. The reaction mixture and MRS broth were incubated at 37 °C for 24 h. All the experiments were repeated twice.

In vitro cholesterol-lowering test

MRS broth (pH7.0) (Difco, USA) was prepared and autoclaved at 121°C for 15 min. Soluble horse serum (Sigma, USA) was added to the prepared MRS broth and filtered through a 0.45 µm Millipore filter. The inoculation volume was 15 µl of provisional probiotic bacterial culture (10^8-10^9 CFU/ml) solution per 1 ml cholesterol-MRS broth, and that was microaerobically incubated at 37°C for 18 h. Uninoculated MRS broth was also incubated at 37°C for 18 h for the control. Following incubation, bacterial cells were removed by centrifugation (3,000 rpm, 10 min), and the culture broth and uninoculated control broth were then assayed for their cholesterol content. The remaining volume of cholesterol in the cholesterol-MRS broth was determined by the method reported by Rudel and Morris with a small modification[41]in which 3 mL of supernatant, 2 mL of 33% (w/v) KOH and 3 ml 96% ethanol were placed in a capped test tube, vortexed for 20 sec and incubated for 15 mins at 60°C in a water bath. After incubation, the mixture was removed and cooled under tap water, then 5 mL of hexane and 3 mL of water were added and vortexed for one min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. To measure the amount of cholesterol, the dye layer is observed at 560 nm. The ability of bacterial strain to remove cholesterolfrom media was calculated as percentage fromthe following equation: $A = 100 - (B/C)\times100$, where A =% of cholesterol removed, B = absorbance of the samplecontaining the cells and C = absorbance of the samplewithout cells. It was observed that, the sample withoutcells has no pellet following centrifugation and cholesterolwas therefore determined in the whole sample.

Results and Discussion

Antimicrobial activity

The strains of LAB did not show antifungal activity against *C. albicans* but some inhibited bacterial indicator strains (*Bacillus subtilis* TISTR 008, *Escherichia coli* TISTR 887, *P. aeruginosa* TISTR 781, *S. aureus* TISTR 517, and *M.luteus* TISTR 884), as tabulated in Table 2.

Table 2 Inhibition of bacteria and yeast by selected lactic acid bacteria (LAB) using cross streak method

LAB strains	Antimicrobial activity[1]	
	Indicator bacterial strains[2]	*Candida albicans*
P. pentosaceus 1 L14/1	+	-
P. acidilactici L25	+	-
Lb. plantarum L26	+	-
Lb. pentosus	+	-
E. faecium N 15	+	-

[1]**Antimicrobial activity: +, supression; -, no suppression**

[2]**Indicator bacterial strains also see Materials and Methods (Microbial inocula).**

Acid resistance and bile tolerance of selected strain of LAB

A selected strains of LAB were isolated from the fermented foods or probiotic products in Thasala, Nakhon Si Thammarat area, southern Thailand. Probiotic bacteria must be resistant to the high acidity of the stomach and high concentration of bile components in the proximalintestine in animal nutrition and in therapy. These characteristics may be observed *in vitro* and can be used for selection of strains[42]. Thus, MRS broth adjusted to pH2.5 or containing 0.3% (w/v) oxgall were used to select acid resistant and bile tolerant LAB isolates. In this study, it was observed that selected LAB tested is sensitive to acid and no survival was observed after exposure to acidified MRS broth of pH 2.5 for 3 h. However, there were 5 strains LAB including two *Pediococcus* spp. (L14/1, L25), two *Lactobacillus* spp. (L26, *Lb. pentosus*), and one *Enterococcus* spp. (N15) showed moderate survival rate (4.25-5.25%) and the final viable bacterial count at pH2.5 or in presence 0.3% oxgall after 3 h of incubation remained at the levels of $>10^4$cfu/ml. The results are showed in Table 3. The data indicated that these strains might survive at the low pH conditions in the stomach (pH2.0 in extreme cases). Unconjugated bile acids, even at lowconcentrations, can inhibit the *in vitro* growthof microorganisms[43]. At 0.3% of bile salt is considered to be a critical concentration for screening for resistant strains[44]. Table 3 showed the survival viable bacteria counts (mean value of log cfu/ml±standard deviation) of selected strains of LAB in brothcontaining 0.3% oxgall. *Lb.plantarum*L26 and*E. faecium* N15 remained at the levels of 10^5cfu/ml after 3 h of incubation in presence of oxgall, thatshowed higher ability to withstand bile concentration of 0.3% oxgall thanthe other strains. This indicates that both selected strains maybe better adapted to tolerate the intestinal bile conditions.

Table 3: Survival of the lactic acid bacteria (LAB) strains at pH2.5 and in the presence of 0.3% oxgall.

LAB strains	pH2.5	0.3% oxgall
P. pentosaceus 1 L14/1	4.25±0.24	4.55±0.21
P. acidilactici L25	4.37±0.26	4.23±0.11
Lb. plantarum L26	4.38±0.41	5.61±0.20
Lb. pentosus	4.32±0.36	4.59±0.27
E. faecium N 15	4.17±0.13	5.32±0.25

Experiments were performed in duplicate, the data are expressed as log cfu/ml mean values± standard deviation

Cholesterol-removing ability

Overmuch cholesterol in the blood and diet is a major risk factor for coronary heart disease. Therefore, decreasing in serum cholesterol levels is important to prevent the disease. The cholesterol-removing ability of probiotic lactobacilli was assessed (Table 4); all tested strains had the ability to remove cholesterol from laboratory media during growth. Among the *Lb. plantarum*L26 removed more cholesterol than other tested strains. Results revealed that addition of bile salts greatly improved the uptake and assimilation of cholesterol from the media. These results agreed with the observations of Pereria and Gibson, 2002[45] that the uptake of cholesterol by LAB was higher in the medium containing 0.4% oxgall. The mechanism by which lactic acid bacteria remove cholesterol from laboratory media has been studied. It has been reported that cholesterol removed by some lactobacilli was due to a disruption of the cholesterol micelles caused by the deconjugationand precipitation of cholesterol with the free bile salts as the pH of the media dropped by acid production during growth[46,47]. However, it was also reported that some strains of *Lactobacillus* spp. incorporated some of the cholesterol into the cellular membrane[48].

Table 4 The percentage of cholesterol removal from media by lactic acid bacteria (LAB) presented by mean±SD

	Cholesterol removal (%)				
LAB strains	**L14/1**	**L25**	**L26**	**N15**	***Lb. pentosus***
Without bile salt	15.17 ± 0.17	15.79 ± 0.17	17.03± 0.84	17.12± 0.17	15.19 ± 0.17
With 0.3% oxgall	18.25 ± 0.51	19.38 ± 0.67	59.52 ± 2.02	64.88±0.51	19.28 ± 0.67

L14/1, *Pediococcus pentosaceus* 1; L25, *P. acidilactici*, L26, *Lactobacillus plantarum*; N15, *Enterococcus faecium*

Conclusion

The selected strains of lactic acid bacteria (LAB) showsantibacterial activity. Only two selected LAB (L26, N15) shows ability to uptake cholesterol from media. The degree of cholesterol uptake depends on isolate. *Lb. plantarum*L26 could be selected for specificpurposes. Indeed the most important factor for removing the cholesterol level is the strain of bacteria. These finding postulate that this strain could be used for reducing high cholesterol levels in patient. However, further studies are required to determine the mechanism(s) involved in the removal of cholesterol by those of LABstrains *in vivo*.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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