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ORIGINAL PAPER

Analysis of functional properties of *Lactobacillus acidophilus*

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Abstract Metabolites from *Lactobacillus acidophilus* were analysed. The results showed that *Lactobacillus acidophilus* Ind-1 and *Lactobacillus acidophilus* Lakcid produced respectively 12.73 g and 13.33 g lactic acid l^{-1} after incubating in skim milk at 37 °C for 36 h; and 2.229 unit and 1.808 unit β-galactosidase l^{-1} in an MRS medium. The proteolytic activity of *Lactobacillus acidophilus* was high and the content of 17 free amino acids in the fermented milk of *Lactobacillus acidophilus* Ind-1 and *Lactobacillus acidophilus* Lakcid was 394.4 mg l^{-1} and 563.2 mg l^{-1}, respectively. Meantime, *Lactobacillus acidophilus* reduced cholesterol level in an MRS medium supplemented with cholesterol. Furthermore, *Lactobacillus acidophilus* Ind-1 and *Lactobacillus acidophilus* Lakcid showed antimicrobial activity against *Bacillus anthracis* and *Escherichia coli.*

Keywords Antimicrobial activity · β-Galactosidase · Cholesterol reduction · *Lactobacillus acidophilus* · Probiotic effects · Protein availability

Introduction

Lactobacillus acidophilus is one of the major species of the natural microflora in animal and human intestines. Lactobacilli, in sufficient quantity, can create special probiotic effects: (1) act as barriers against pathogenic organisms; (2) assist in lactose digestion; (3) improve he nutritional level of food products; (4) potentially enhance the development of host's immune response; and (5) reduce cholesterol level (Fuller 1991; Gilliland 1990a). Thus, dairy products containing *Lactobacillus acidophilus* have an increased value as a dietary supplement (Zhao et al. 2001, 2005). Generally, the ideal probiotic microorganism would be one which could go through gastrointestinal environment and establish itself permanently in the intestine. When present in a food product, clinical evidence for any stated health effect and laboratory verification of important parameters for physiological function should be considered. Some clinical trials have shown promising potentials that *Lactobacillus* can be used to treat disorders associated with intestinal disturbances due to pathogens (Clements et al. 1981, 1983). Oksanen et al. (1990) treated with lactobacilli 756 Finnish tourists who traveled to Turkey and suffered from diarrhea. He observed a decrease in diarrhea frequency from 71% to 43% by the treatment with lactobacilli. Suarez and Savaiano (1997) reported that fermented milk, especially yogurt greatly improves lactose digestion for persons with a low intestinal lactase activity, due to the existence of the bacterial enzyme β-galactosidase. This explained why these individuals could tolerate yogurt very well while they developed symptoms of lactose-intolerance after ingesting similar amounts of milk. Others reported the enhancement of host immune response in experimental animals like mice (Perdigon 1989) and reduction of the cholesterol level in pigs (Lin 1989). However, the physiological significance of these tests for humans and evidence supporting these claims

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have not yet been thoroughly established. Yogurt is very popular because of its pleasant flavor and special nutritional properties. However, yogurt starters used nowadays are dominantly *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. These bacteria produce lactic acid, acetic acid, bacteriocins, and others, but cannot survive in the gastroenteric environment and colonize the intestines to promote human health. According to the literature, a minimum concentration of *Lactobacillus acidophilus*, 10^5 c.f.u. (colony-forming units) to 10^6 c.f.u. per ml, or per gram product is needed to have therapeutic benefits (Kurmann and Rasic 1988). Nevertheless, evidence supporting probiotic claims of *Lactobacillus acidophilus* is very slight. This means that more study of the probiotic characteristics of *Lactobacillus acidophilus* is needed to give an explanation for the positive effect on human and animal health. In this study we aimed at: (1) examining some metabolites produced by *Lactobacillus acidophilus* (such as lactic acid, β-galactosidase, 17 free amino acids); (2) testing cholesterol reduction and antimicrobial activity on pathogenic organisms.

Materials and methods

Microorganisms

Lactobacillus acidophilus Ind-1 (La-1) and *Lactobacillus acidophilus* Lakcid (La-2) were obtained from the Department of Food Science, Henan Institute of Science and Technology. *Bacillus anthracis* and *Escherichia coli* were obtained from Sanitation and Anti-epidemic Station of Xinxiang City, Henan Province, China.

Media

La-1 and La-2 were incubated in skim milk at 37 °C for 36 h to measure the concentration of lactic acid and free amino acids, and incubated in deMan Rogosa and Sharpe (MRS) medium supplemented with 4%(w/v) lactose as described by Zhao et al. (2003) to determine β-galactosidase, and with different content cholesterol to test cholesterol reduction. *Bacillus anthracis* and *Escherichia coli* were grown on nutrient culture medium (peptone 5.0 g, meat concentrate 3.0 g, yeast extract 1.0 g, agar 10.0 g, in 1000 ml water, pH 6.8).

Assay for lactic acid

Sterilized skim milk was inoculated with 10% (v/v) liquid culture of La-1 and La-2 aseptically, and then incubated at 37 °C for 36 h. The fermented skim milk was extracted with 80% (v/v) ethanol, at 75 °C for 15 min and centrifuged (RC-5C, Low Temperature Superspeed Centrifuge, America Sorvall@ instruments Du Pont Company) at 1,247 *g* for 15 min, followed by the collection of the supernatant. The supernatant was centrifuged again at 31,186 *g* for 15 min, followed by ultrafiltration (0.45 μm). The ultrafiltrate (10 μl) was assayed by HPLC at 275 nm (2996PDA Detector, Waters Empower HPLC System, Waters Co. U.S.A). The injected doses of standard samples of lactic acid, oxalic acid, citric acid, tartaric acid, malic acid, acetic acid and succinic acid were 0.200, 0.005, 0.120, 0.005, 0.150, 0.200, 0.180 g l^{-1}, respectively, according to the sensibility of forming the wave front. By comparing with the standard time of the forming peak, and according to the relative peak area, the amount of every organic acid above was calculated (Ning 2001).

Assay for β-galactosidase

Lactose can be decomposed into one glucose and one galactose and is used as substrate for β-galactosidase determination. The structure of the colourless *O*-nitrophenyl-β-D-galactopyranoside (ONPG) is similar to that of lactose, and also can be decomposed by β-galactosidase into yellowish *O*-nitrophenol (ONP). The amount of ONP released can be measured to determine β-galactosidase activity by spectrophotometer (Qian 1992). To collect cells, 50 ml of MRS culture was centrifuged at 3,600 *g* for 15 min. The precipitate was washed twice with 0.05 M NaH_2PO_4 buffer, and 10 ml NaH_2PO_4 buffer was added to make the cell suspension thereafter. Then 100 μl solvent mixture (1%SDS(w/v):chloroform = 1:10, by vol.) was added and incubated at 37 °C for 30 min, and followed by centrifugation (4,000 *g*, 10 min). The supernatant (1 ml) mixed with 4 ml 0.005 M ONPG was incubated at 37 °C for 20 min. The reaction was stopped by adding 5 ml 0.5 M cold Na_2CO_3. The amount of ONP released was measured by spectrophotometry at 420 nm (U/V-1100 u.v. spectrophotometer, Beijing Ruili Analytic Facility Company, Beijing, China). The units of lactase activity utilizing ONPG as substrate was expressed as the formation of micromole (μmol) of ONP from ONPG per min per liter sample. That is, 1 unit l^{-1} β-galactosidase equalled to 1 μmol ONP from ONPG per min per liter sample, relatively (Qian 1992). The growth curves of La-1 and La-2 were followed by measuring the optical density (O.D.) at 600 nm in MRS (Braun et al. 1981).

Assay for free amino acid

About 5 ml of fermented skim milk (the same method as for lactic acid described above) mixed with 10 ml 10% (v/v) metaphosphate, was centrifuged at 17,542 *g* for 15 min. Subsequently, 1 ml supernatant was mixed with 1 ml of citric acid buffer (pH 2.2). The mixture (50 μl) was collected and 17 free amino acids were quantitatively determined by an Amino Acid Analytic Facility (121MB, America Beckman Company) (Zhao et al. 2002; Ning 2001).

Test of cholesterol reduction

The normal human serum cholesterol level is between 2.33 $mmol^{-1}$ and 5.69 $mmol^{-1}$ (the criteria normally used to judge hypercholesteremia), MRS medium was supplemented with cholesterol to reach 7.755 $mmol^{-1}$, 5.170 $mmol^{-1}$ and 2.585 $mmol^{-1}$, respectively, then sterilized at 121 °C for 15 min, cooled to 37 °C, inoculated with liquid culture (10%, v/v), and incubated at 37 °C for 24 h. Culture broth (1.5 ml) was centrifuged at 900 *g* for 10 min, and 0.5 ml supernatant was added with 3.0 ml color reagent, containing 0.03 ml cholesterol oxidase (20 $unitml^{-1}$), 8 mg cholesterol esterase (40 $unitg^{-1}$) and 0.1 mg peroxidase (Reinheit Zahl = 3 mg^{-1}), and kept at 37 °C in a water bath for 10 min. The amount of cholesterol was determined by the so-called CHOD-PAP method with spectrophotometer at 500 nm (Gilliland and Walker 1990b; Ning 2001). The experiments were done in duplicate and the control was done without adding liquid culture.

Antimicrobial activity against *B. anthracis* and *E. coli*

Bacillus anthracis and *E. coli* were inoculated by plate spreading under aseptic conditions on nutrient culture medium plates, cultivated at 37 °C for 1 h to make the colony fixed, then 3 discs with a diameter of 3 mm in each plate were stabbed and 3 holes remained. MRS liquid medium previously inoculated by 10% (v/v) La-1 and La-2 was incubated at 37 °C for 36 h to make the liquid culture reach 10^6 c.f.u. ml^{-1} La-1 and La-2, after which it was injected into the 3 holes. After 24 h incubation at 37 °C, antimicrobial activity was measured by agar diffusion method as modified by Kirby-Bauer, recommended by World Health Organization (WHO) in 1997 (Ling and Dong 1999; Li et al. 2003; Mo et al. 2005).

Results and discussion

Organic acid produced by Lactobacillus acidophilus

Levels and kinds of accumulated organic acids by La-1 and La-2 were shown in Table 1. The amount of lactic acid of La-1 and La-2 was 12.73 g l^{-1} and 13.33 g l^{-1}, oxalic acid was 0.70 g l^{-1} and 0.45 g l^{-1}, and citric acid was 1.02 g l^{-1} and 0.75 g l^{-1}, respectively. Trace amounts of malic acid were produced, while no tartaric acid, acetic acid and succinic acid were detected. According to the amount of lactic acid, carbohydrate fermentation of *Lactobacillus acidophilus* in skim milk was homolactic, with lactic acid being more than 90% of the end products. This meant that the test strains could increase lactose utilization and hence lower lactose concentration in milk. This was probably one of the reasons why *Lactobacillus acidophilus* strains were able to increase lactose tolerance for individuals, and consequently, they could be used in probiotic preparations.

β-galactosidase activity in MRS

Experiments had revealed that β-galactosidase could improve lactose utilization for lactose-intolerant persons (Jiang et al. 1996). Lactose is taken up by a specific permease inside bacterial cells and is split by β-galactosidase into glucose and D-galactose (Kandler 1983). Figure 1 gives the levels of β-galactosidase activity and the relationships between the microbial growth and the production of β-galactosidase by La-1 and La-2 at certain incubation times. Compared with the growth curves of La-1 and La-2 (Fig. 1), the change of β-galactosidase activity had a different trend. The enzyme activity of La-1 and La-2 increased with the cultivation time and reached its peak of 2.229 unit l^{-1} and 1.808 unit l^{-1} at 36 h, respectively. After 36 h, levels of β-galactosidase activity declined and at 60 h had fallen to 1.387 unit l^{-1} and 0.922 unit l^{-1}, respectively for La-1 and La-2. Reportedly, other strains used

Table 1 Types and quantity of organic acid formed by *Lactobacillus acidophilus*

Organic acid	Quantity (g/l)	
	La-1	La-2
Oxalic acid	0.70	0.45
Tartaric acid	n.d.[a]	n.d.
Malic acid	trace	trace
Lactic acid	12.73	13.33
Acetic acid	n.d.	n.d.
Citric acid	1.02	0.75
Succinic acid	n.d.	n.d.

[a]n.d. = not detected

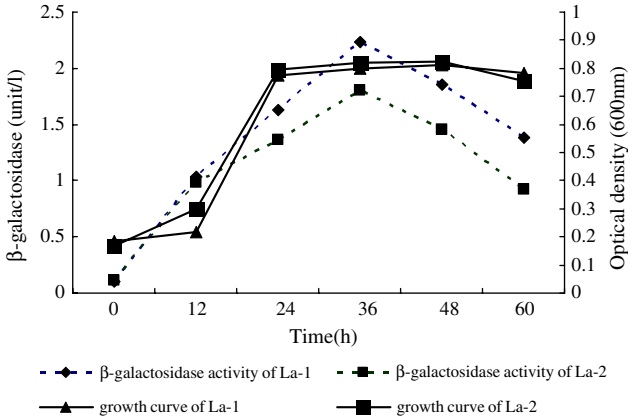

Fig. 1 Growth and *β*-galactosidase production by *Lactobacillus acidophilus*

in fermented milk such as *Lactococcus lactis* subsp. *lactis E* only produced 0.146 unit *β*-galactosidase l^{-1} and *Lactococcus lactis* subsp. *lactis C10* produced 0.331 unit *β*-galactosidase l^{-1} (Pochart et al. 1989). In our study, La-1 and La-2 had much higher *β*-galactosidase activity than that of above reported strains. This confirmed again why *Lactobacillus acidophilus* strains were able to improve lactose tolerance for individuals.

Production of free amino acids in fermented skim milk

Through fermentation, the amount of amino acids in skim milk increased greatly compared with unfermented skim milk (as shown in Fig. 2), especially glutamic acid, proline, asparagine, valine, leucine, tyrosine and arginine. The total amount of amino acids of fermented skim milk by La-1 and La-2 was 394.4 mg l^{-1} and 563.2 mg l^{-1}, respectively. Unfermented milk had only 43.2 mg l^{-1} and yogurt fermented by a routine starter culture with a mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* had 151.1 mg free amino acids l^{-1}. In our study, La-1 and La-2 indeed had the ability to hydrolyze milk proteins to free amino acids. This also indicated that different strains showed different abilities to hydrolyze milk protein. As protein availability in milk was evaluated by its free amino acid content, fermentation by La-1 and La-2 improved the quality and availability of milk protein.

Assay for cholesterol reduction

Figure 3 (a and b) show the cholesterol reduction ability of La-1 and La-2 in MRS medium supplemented

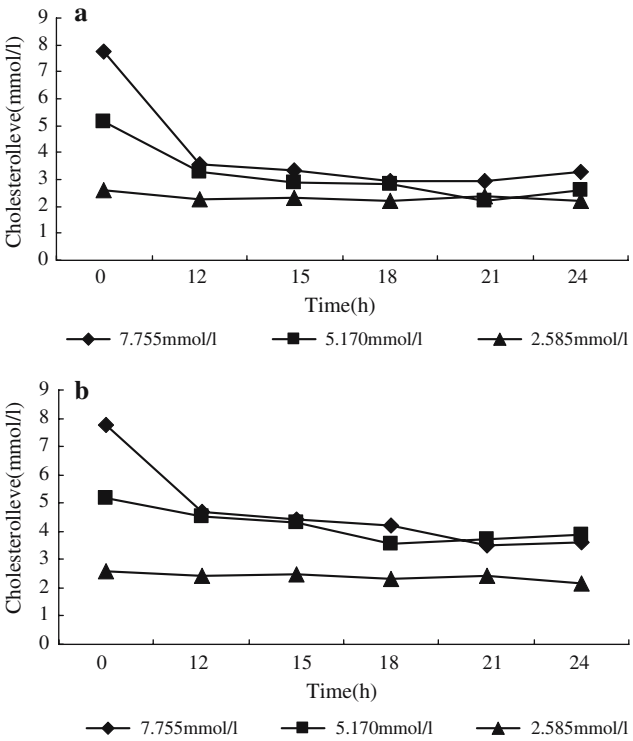

Fig. 3 (**a**) The cholesterol reduction ability of La-1 (**b**) The cholesterol reduction ability of La-2

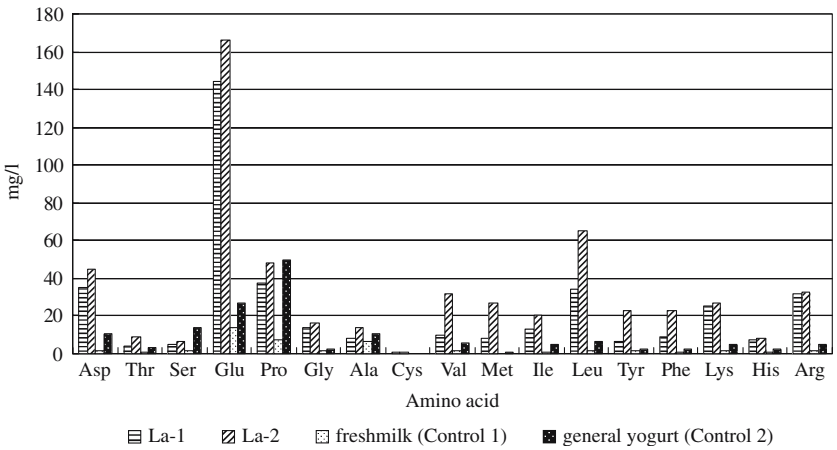

Fig. 2 Content of free amino acid in fermented milk of La-1 and La-2

with different cholesterol level, namely 7.755 mmol l^{-1}, 5.170 mmol l^{-1} and 2.585 mmol l^{-1}, respectively.

Figures 3a and b show that La-1 and La-2 could reduce cholesterol level if the initiation concentration was 7.755 $mmol^{-1}$ and 5.170 $mmol^{-1}$ in MRS medium, no effect on that of 2.585 $mmol^{-1}$. From Figure 3, we also could see that the reduction of cholesterol level was mainly made within 12 h at the beginning, and with the time on, the changes were very small. After 24 h, the cholesterol levels were all below the critical level 5.69 $mmol^{-1}$, a critical point above which one will be judged to have hypercholesteremia. There was no any change in control test. This implies that *Lactobacillus acidophilus* was effective in reducing cholesterol level in MRS medium. However, these were only *in vitro*. Further tests on animals or humans should be done to prove whether *Lactobacillus acidophilus* could really reduce serum cholesterol level.

Antimicrobial effects on pathogenic bacteria

As shown in Figs. 4a and b, the zones of growth inhibition on *B. anthracis* and *E. coli* by La-1 were 16–18 mm and 16–20 mm, and by La-2 were 20–22 mm and 18–20 mm, respectively. This meant that La-1 and La-2 had antimicrobial effect against *B. anthracis* and *E. coli*. They could act as an antibacterial agent against certain Gram-positive and -negative pathogenic bacteria. The mechanism of the antimicrobial effect was very complicated. These activities might have been resulted from some organic acids, or from other metabolites, such as bacteriocin, analogous bacteriocin and H_2O_2, etc. (Schilliager et al. 1997), as well as *Bifidobacterium* and *Lactobacillus* acting as probiotic microorganisms with antagonistic action toward intestinal pathogens (Hotta et al. 1987; Isolauri et al. 1991).

Conclusions

Strains La-1 and La-2 had clear probiotic properties including acidification, β-galactosidase activity, proteolytic activity, cholesterol reduction, and antagonistic action on *B. anthracis* and *E. coli*. This confirmed that *Lactobacillus acidophilus* could be used as a potential probiotic dietary supplement.

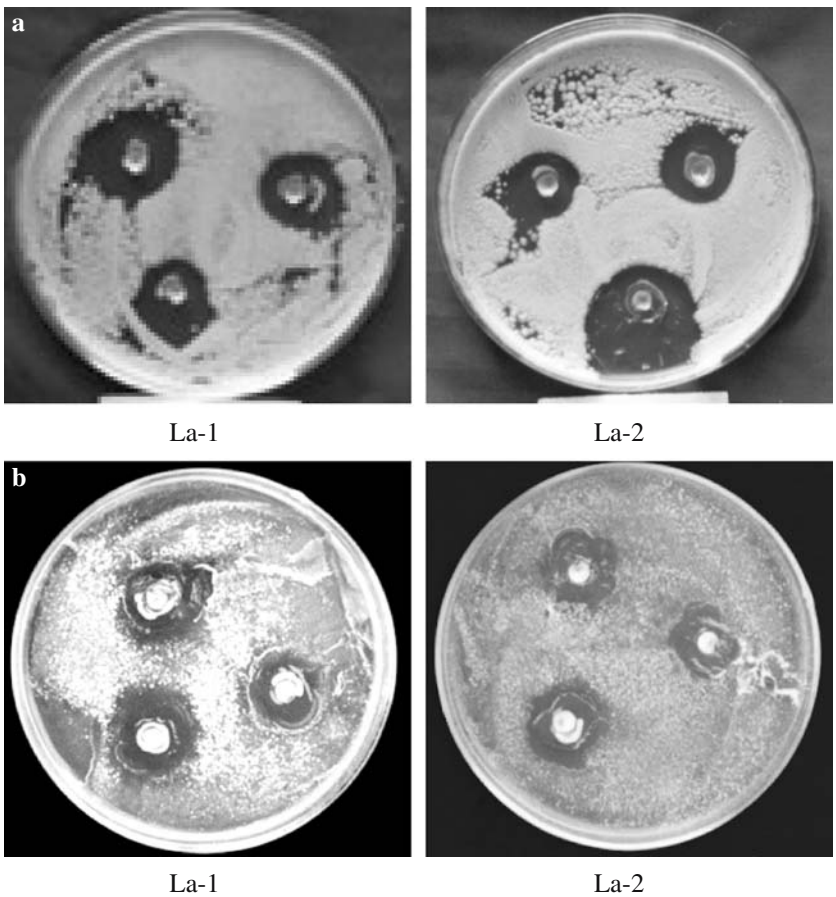

Fig. 4 (**a**) Antibacterial activity of La-1 and La-2 on *B. anthracis* (**b**) Antibacterial activity of La-1 and La-2 on *E. coli*

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