

Two Different Methods for Screening of Bile Salt Hydrolase Activity in *Lactobacillus* Strains

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Abstract

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Bile salt hydrolase (BSH) activity of intestinal bacteria (including lactobacilli) is one of the indirect ways of decreasing a cholesterol level in human body. Tested *Lactobacillus* strains were isolated from various sources (faeces of fully breast-fed infants, cow's colostrum, cow's raw milk cheeses, and cow's raw milk) and identified by genotypic and phenotypic methods. All strains, including three commercial probiotic strains and six culture collection strains, were subsequently tested for their BSH activity via two methods – thin layer chromatography (TLC) and plate assay. Among all the 59 *Lactobacillus* strains, 15 strains were shown to be BSH positive by TLC method and only 8 of them by plate assay. Most of the BSH positive strains (9 strains) were isolated from faeces. Differences between BSH activities for sodium salts of cholytaurine and cholyglycine were demonstrated. The TLC method was shown to be more sensitive compared to the plate assay for BSH activity determination.

Keywords: *Lactobacillus*; bile salt hydrolase activity

Historically, lactobacilli have been used for a long time in food technology, mainly in the production of dairy products and are considered to be Generally Recognised as Safe (GRAS) by the World Health Organization (FAO/WHO 2002). Lactobacilli can be safely added into foods as food additives. Lactobacilli have multiple beneficial properties, including antimicrobial activity (LEE *et al.* 2011; ZHANG *et al.* 2011), immunostimulation (CROSS 2002), antimutagenicity (CALDINI *et al.* 2005), anticarcinogenicity (BURNS & ROWLAND 2000) or bile salt hydrolysing (DASHKEVICZ & FEIGHNER 1989; AHN *et al.* 2003; LIONG & SHAH 2005a; SRIDEVI *et al.* 2009), which makes lactobacilli valuable as probiotic microorganisms.

Cholyglycine hydrolase (EC 3.5.1.24), also known as bile salt hydrolase (BSH), hydrolyses bile salts to form glycine or taurine, as well as steroid core (LIONG & SHAH 2005a; BEGLEY *et al.* 2006). Bile salts recirculate within human bodies. Hydrolysed bile salts compared to non-hydrolysed ones are less absorbed

in the human intestine, leaving more free bile acids to be excreted out of human bodies via faeces (BEGLEY *et al.* 2006). Increased excretions of bile salts, therefore, decrease the total amount of bile salts available in human bodies. The lost bile salts can be replenished via *de novo* synthesis from cholesterol, which could subsequently reduce the level of serum cholesterol in human body (BEGLEY *et al.* 2006). BSH activity has been found in several bacterial genera, i.e. *Lactobacillus* (DASHKEVICZ & FEIGHNER 1989; AHN *et al.* 2003; LIONG & SHAH 2005a; SRIDEVI *et al.* 2009; LEE *et al.* 2011), *Bifidobacterium* (LIONG & SHAH 2005b; NORIEGA *et al.* 2006), *Enterococcus* (FRANZ *et al.* 2001), *Clostridium* (KISHINAKA *et al.* 1994), *Bacteroides* (STELLWAG & HYLEMON 1976), etc. However, based on TANAKA *et al.* (1999), not all strains of lactobacilli isolated from gastrointestinal tracts possess BSH activity, and some lactobacilli and bifidobacteria isolated from environments other than gastrointestinal tracts may also have BSH activity.

The aim of this study was to isolate and identify lactobacilli obtained from different sources and screen them for BSH activity using two methods: thin layer chromatography (TLC) and plate assay.

MATERIAL AND METHODS

Bacterial strains. In total 59 lactobacilli strains were used in this study. 27 strains of lactobacilli were isolated from faeces of fully breast-fed infants at Czech University of Life Science, Prague, Czech Republic and kindly provided for this study. Their identification as well as the isolation and identification of strains from colostrum (4), cow's raw milk (7), and cheeses made of cow's raw milk (12) were done at the University of Chemistry and Technology, Prague, Czech Republic. Six culture collection strains (*L. acidophilus* CCDM 151, *L. rhamnosus* CCDM 150, *L. casei* CCDM 198, *L. acidophilus* CCDM 406 Laktoflora[®]; Milcom, Prague, Czech Republic; *L. acidophilus* ATCC[®] 4356TM, *L. acidophilus* ATCC[®] 314TM; Oxoid, Basingstoke, Hampshire, UK), three commercial probiotic strains (*L. acidophilus* LAFTI[®] L10, *L. casei* LAFTI[®] L26; DSM Food Specialties, JH Heerlen, the Netherlands, *L. acidophilus* LA-5; Ch. Hansen, Hørsholm, Denmark) and *Escherichia coli* CNCTC 6859 (Czech National Collection of Type Cultures, Prague, Czech Republic) were also included in this study.

Isolation of bacteria. The strains were isolated from Rogosa agar (pH 5.5; Merck, Darmstadt, Germany) at 37°C under an atmosphere of 5% v/v CO₂. Selected colonies were streaked onto MRS agar (pH 5.6; Oxoid, Basingstoke, Hampshire, UK). Subsequent cultivations were carried out in the same conditions and individual selected colonies were transferred into MRS broth (pH 5.6; Oxoid).

Identification of bacteria. Isolated bacteria were identified thereafter, using phenotypic methods which included Gram staining, bacteria morphology, and catalase activity. The Gram-positive, catalase negative, rod-shaped bacteria with proved BSH activity were further identified by genotypic methods. The first method was PCR for identification of the genus *Lactobacillus* (VENTURA *et al.* 2000) and the second one was 16S rRNA gene sequence analysis for determination of *Lactobacillus* species (GODON *et al.* 1997). The identification of isolated strains was achieved by comparing the results with the bacterial genome database of NCBI.

Cultivation of bacteria. Cultivations of *Lactobacillus* strains were carried out at 37°C for 18 h under an atmosphere of 5% v/v CO₂ in MRS broth (pH 5.6; Oxoid). *Escherichia coli* CNCTC 6859 was cultivated in Brain Heart Infusion (BHI) broth (pH 7; Himedia, Mumbai, India) in the same conditions as *Lactobacillus* strains.

BSH activity – Plate assay. The protocol for bile salt hydrolase activity measurement was based upon DASHKEVICZ and FEIGNER (1989) and AHN *et al.* (2003) with modifications. Soft MRS agar (pH 5.6; Oxoid), which contained MRS broth (52.5 g/l; Oxoid), bacteriological agar (7.5 g/l; Oxoid), bile salts (0.3% w/v; Ox Bile, Himedia, India), and CaCl₂ (0.375 g/l; Lach-Ner, Neratovice, Czech Republic), was used. Petri dishes with agar were incubated under an anaerobic atmosphere at 37°C for 48 hours. Various lactobacilli strains (10 µl), which had been cultivated for 18 h under an atmosphere of 5% v/v CO₂, were inoculated on MRS agar by puncturing into the agar. Subsequent cultivation of the agar media containing bacterial strains was carried out at 37°C for 72 h under an anaerobic atmosphere. Visible halos around the punctures indicate the positive BSH activity of the strains. Results were then assessed by measuring the diameters of halos. Lactobacilli strains grown on MRS agar without bile salts were used as the negative control. Measurements were repeated three times.

BSH activity – thin layer chromatography (TLC). According to GUO *et al.* (2011), lactobacilli strains were cultivated in MRS broth (5 ml; Oxoid) at 37°C for 18 h, followed by centrifugation at 9000 g at 4°C for 10 minutes. The centrifuged samples were then washed with 2 ml of physiological solution. After washing, 5 ml of physiological solution was added to the cell pellets of lactobacilli. Bacterial suspension (1 ml) was mixed with reaction mix (1 ml). The reaction mix contained MRS broth (Oxoid), with either sodium salt of cholyglycine (Na-GCA, 0.3% w/v) or sodium salt of cholytaurine (Na-TCA, 0.3% w/v; both St. Louis, USA) in phosphate buffer (0.1 mol/l), which gave a final pH of 6.5. The strains were then cultivated at 37°C for 8 h under an atmosphere of 5% v/v CO₂. After the cultivation, the samples were vacuum evaporated and the residuals were subsequently dissolved in 1 ml of methanol (Lach-Ner, Neratovice, Czech Republic), followed by centrifugation at 14 000 g at 4°C for 1 minute. The supernatants were spotted onto the baselines on silica gel plates (10 × 10 cm, TLC silica gel 60 F₂₅₄; Merck), along with spotting of standard solutions,

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which were cholic acid (CA) in methanol (5 mmol/l), Na-TCA in methanol (5 mmol/l) and Na-GCA in methanol (5 mmol/l; all Sigma-Aldrich, St. Louis, USA). The mobile phase contained isoamyl acetate (Merck, Darmstadt, Germany), propionic acid (Fluka, Buchs, Switzerland), *n*-propanol (Penta, Prague, Czech Republic) and water at a ratio of 40:30:20:10. The mobile phase was allowed to migrate along TLC plates for 30 minutes. The plates were then dried and sprayed with 10% w/v solution of phosphomolybdic acid (Lachema, Brno, Czech Republic) in ethanol, followed by drying with hot air. Cholic acid was liberated from bile salts by BSH positive strains. Results of TLC were evaluated by comparing with standards. Measurements were repeated three times.

RESULTS AND DISCUSSION

Survival of probiotics while passing through the gastrointestinal tract is of great importance for maintaining the health benefits of probiotics (ELLI *et al.* 2006). Many factors can affect the survival of bacteria in the gastrointestinal tract, i.e. pH of the environment, time of exposure to certain pH, presence and concentration of bile salts, etc. In the environment of small intestine, presence of bile salts is the most undesirable factor for the survival of probiotics (BEZKOROVAINY 2001). Therefore, probiotic lactobacilli with BSH enzymes, which hydrolyse bile salts, were found to have a higher possibility of survival in the gastrointestinal tract (DU TOIT *et al.* 1998). Furthermore, BSH activity is one of the indirect possibilities how to decrease a cholesterol level in human body (BEGLEY *et al.* 2006).

In this study, 59 Gram-positive, rod-shaped, and catalase negative strains were isolated, of which 27 strains were isolated from faeces of fully breast-fed infants (from 5 to 207 days old), 4 strains were

Table 1. Origin of strains used in this study with the ratio between bile salt hydrolase (BSH) positive and negative strains (TLC method)

Strain origin	Total	BSH	
		positive	negative
Faeces of fully breast-fed infants	27	9	18
Raw milk	7	1	6
Colostrum	4	0	4
Cheeses from raw milk	12	0	12
Culture collection strains	6	4	2
Commercial probiotic strains	3	1	2

isolated from cow's colostrum, 12 strains from cheeses made from cow's raw milk, and 7 strains from cow's raw milk. All the isolates were confirmed by PCR identification as lactobacilli.

All of 50 newly isolated strains, 6 collection strains and 3 commercial probiotic strains were screened for their BSH activity by use of plate assay and TLC. *L. acidophilus* ATCC 4356 was used as a comparative BSH positive strain (DASHKEVICZ & FEIGNER 1989; LIONG & SHAH 2005a), and *Escherichia coli* CNCTC 6859 was used as the BSH negative strain (BEGLEY *et al.* 2006; GUO *et al.* 2011). The results are summarised in Table 1. It was found that 15 out of the 59 strains were demonstrated to have BSH activity using TLC method.

In Table 2 the results from TLC method and plate assay are compared for BSH positive strains. The strain specification is also included. Only 8 of these 15 positive strains were confirmed to be BSH positive using the plate assay. All strains which were found BSH negative by TLC method were also confirmed as negative by the plate assay. TLC method was shown to be more sensitive compared to the plate assay for BSH activity determination. Figure 1 shows examples of TLC plates, where hydrolysis of Na-GCA and Na-TCA is visible. Figure 2 demonstrates precipitated zones around BSH positive lactobacilli strains on the Petri dish which varied among the strains from 15 mm to 32 mm.

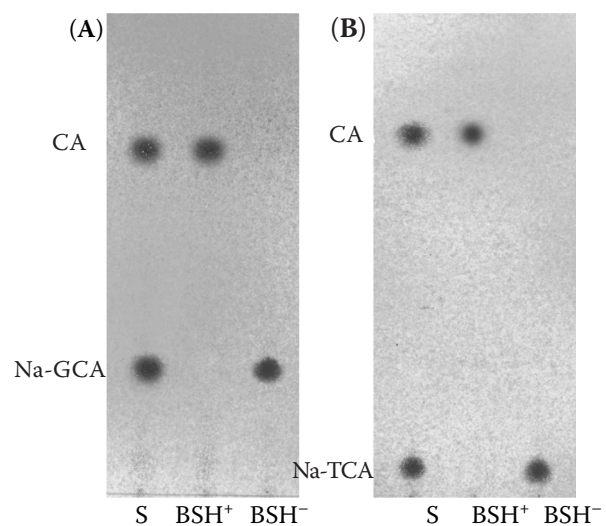


Figure 1. Example of TLC method for bile salt hydrolase (BSH) activity determination: (A) BSH activity for sodium salt of cholyglycine (Na-GCA), (B) BSH activity for sodium salt of cholytaurine (Na-TCA)

S – standard of Na-TCA, Na-GCA, and cholic acid (CA) in 0.1 mol/l phosphate buffer (0.3% w/v); BSH⁺ – BSH positive strain, BSH⁻ – BSH negative strain

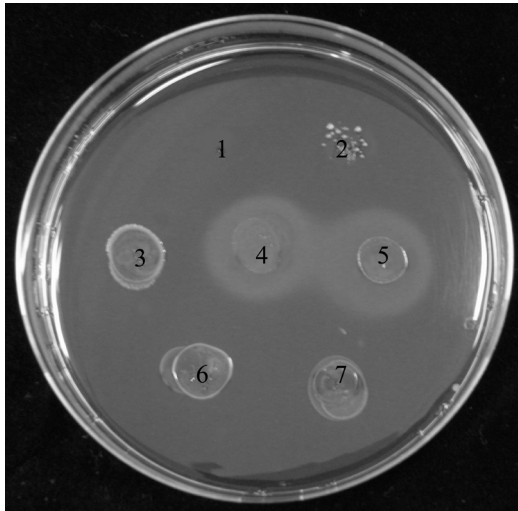


Figure 2. Plate assay for bile salt hydrolase (BSH) activity determination in soft MRS agar with Ox Bile (0.3% w/v) and CaCl_2 (0.37 g/l)

1 – *E. coli* CNCTC 6859, 2 – *L. casei* JN, 3 – *L. brevis* S1, 4 – *L. gasseri* VI, 5 – *L. acidophilus* ATCC 4356, 6 – *L. gasseri* Z3, 7 – *L. rhamnosus* AN

It was demonstrated in this study that most of the BSH positive strains (9 out of 15) were isolated from faeces. It is in accordance with the statement of TANAKA *et al.* (1999) that lactobacilli strains isolated

from the gastrointestinal tract are more likely to be BSH positive, as compared to those without exposures to bile salts. Other BSH positive lactobacilli were the culture collection of strains (*L. acidophilus* ATCC 314, *L. acidophilus* ATCC 4356, *L. acidophilus* CCDM 151, and *L. acidophilus* CCDM 406), commercial probiotic strain *L. acidophilus* LA-5 and one strain from cow's raw milk (*L. brevis* S1). The rest of the strains from cow's raw milk, cheeses, and cow's colostrum did not show any BSH activity, nor did the commercial probiotic strains of *L. acidophilus* LAFTI L10 and *L. casei* LAFTI L26, and collection strains of *L. rhamnosus* CCDM 150 and *L. casei* CCDM 198.

Moreover, as demonstrated in the study by RIDLON *et al.* (2006), *Lactobacillus* spp. may perform different BSH activities in the presence of different bile salts. Specifically, bile salts formed from glycine are more readily to be hydrolysed than bile salts formed from taurine (SUVARNA & BOBY 2005; BEGLEY *et al.* 2006). CORZO and GILLILAND (1999) showed in their study that three strains of *L. acidophilus* performed a higher hydrolysis rate on the sodium salt of cholylglycine than on cholyltaurine. In this study, *L. casei* JN from faeces and *L. brevis* S1 from cow's raw milk did not show any BSH activity on Na-TCA whereas *L. rhamnosus* AN from faeces had no BSH activity

Table 2. Strain specification and the results of plate assay and TLC method of bile salt hydrolase (BSH) positive lactobacilli

Species	Origin	Plate assay (mm) ²	TLC	
			Na-GCA	Na-TCA
<i>L. casei</i> JN	faeces (6 days) ¹	0	P	N
<i>L. gasseri</i> VI	faeces (6 days) ¹	24	P	P
<i>L. gasseri</i> Z2	faeces (131 days) ¹	20	P	P
<i>L. gasseri</i> LBA	faeces (139 days) ¹	24	P	P
<i>L. gasseri</i> Z3	faeces (62 days) ¹	0	P	P
<i>L. gasseri</i> R	faeces (19 days) ¹	15	P	P
<i>L. gasseri</i> ZU 11	faeces (5 days) ¹	0	P	P
<i>L. gasseri</i> Z1	faeces (42 days) ¹	0	P	P
<i>L. rhamnosus</i> AN	faeces (48 days) ¹	0	N	P
<i>L. brevis</i> S1	cow's raw milk	0	P	N
<i>L. acidophilus</i> ATCC 4356	Oxoid	20	P	P
<i>L. acidophilus</i> ATCC 314	Oxoid	30	P	P
<i>L. acidophilus</i> CCDM 151	Laktoflora [®] , Milcom	0	P	P
<i>L. acidophilus</i> CCDM 406	Laktoflora [®] , Milcom	25	P	P
<i>L. acidophilus</i> LA-5	Ch. Hansen	32	P	P

¹faeces of fully breast-fed infants with the infants' age; ²diameter of the zone formed; Na-GCA – sodium salt of cholylglycine; Na-TCA – sodium salt of cholyltaurine; P – positive reaction; N – negative reaction

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on Na-GCA. The rest of the strains were shown to be BSH positive on both salts.

CONCLUSION

Bile salt hydrolase activity of probiotic lactobacilli is more often found in strains isolated from the gastrointestinal tract, where bile salts are present, rather than in strains from the non-bile salt environment. However, some strains isolated from the non-bile salt environment may also possess BSH activity. Also, each lactobacillus strain has its distinct specificity towards bile salts. Moreover, salts of cholyglycine are more readily to be hydrolysed by lactobacilli than salts of cholytaurine. In terms of the methods of BSH activity assessment, we found that the TLC method is easier to evaluate and more sensitive compared to the plate assay.

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