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α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk

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Abstract

The metabolic activities of *Lactobacillus acidophilus* (LAFTI® L10 and La4962) *Bifidobacterium* (*lactis* LAFTI® B94 and *longum* Bl536), *Lactobacillus casei* (LAFTI® L26 and Lc279), *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 and *Streptococuss thermophilus* St1342 were assessed in soymilk. Strains were initially analyzed for α-galactosidase activity and organic acid production in MRS broth at 37 °C. Consequently, soymilk was fermented with each strain and cell growth, production of organic acid, metabolism of oligosaccharides and proteolytic and ACE-inhibitory activities were assessed during 48 h of incubation at 42 °C. All strains exhibited variable α-galactosidase activity, with *Bifidobacterium lactis* B94 showing the highest activity. The oligosaccharide metabolism depended on α-galactosidase activity. *B. lactis* B94, *S. thermophilus* St1342 and *L. acidophilus* La4962 reduced raffinose substantially by 77.4%, 64.5% and 55.9%, respectively. All strains reached the desired therapeutic level of 10^8 cfu/ml in soymilk after 48 h at 42 °C. The hydrolysis of protein in soymilk likely depended on strain ($P < 0.0001$) and time ($P < 0.0001$). The strains also released bioactive peptides with ACE-inhibitory activities between 17% and 43%.

Keywords: Fermented soymilk; Raffinose; Stachyose; α-Galactosidase activity; ACE-inhibitory activity

1. Introduction

Soy-based foods may provide a range of health benefits to consumers due to their hypolipidemic, anticholesterolemic and antiatherogenic properties, and reduced allergenicity (Favaro Trindade, Terzi, Turgo, Della Modesta, & Couri, 2001). They also contain isoflavones, which have been linked to reduced risk of most hormone-associated health disorders (Kurzer, 2000). However, consumption of soymilk is hindered, due to the presence of unpleasant off-flavours carried over from soy beans. These characteristic flavours are caused by *n*-hexanal and -pentanal, which occur in beans as a product of breakdown of unsaturated fatty acids (Arai, Suzuky, Fujimake, & Sakurai, 1996; Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998). In addition to these aldehydes, soymilk contains various oligosaccharides, including raffinose and stachyose, that may cause a gastrointestinal discomfort to consumers (Tsangalis & Shah, 2004).

Raffinose and stachyose are α-galactosides of sucrose, comprising three and four monomeric units, respectively, and are non-digestible in the gut due to the absence of α-galactosidase in the human intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine, where they are metabolized by bacteria that possess this enzyme, resulting in the production of gases (Tsangalis & Shah, 2004). This problem could be alleviated by using a specific enzyme, α-galactosidase, or an organism that possesses high α-galactosidase activity, to minimize the content of flatulence-causing oligosaccharides in the product (Scalabrini et al., 1998). Several

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Bifidobacterium strains have been reported to produce varying levels of α-galactosidase, which metabolize α-galactosyl oligosaccharides in soymilk (Scalabrini et al., 1998). Soymilk is a good medium for growing *Bifidobacterium* because it contains oligosaccharides that are fermented by most of the strains belonging to this genus (Liu, 1997; Scalabrini et al., 1998).

Bifidobacterium spp., *Lactobacillus acidophilus*, and *L. casei*, have been associated with health-promoting effects and are classified as probiotic organisms since they are thought to improve the microbial balance in the human gastrointestinal tract (GIT) (Schrezenmeir & de Vrese, 2001). Health benefits attributed to probiotics include antimicrobial, antimutagenic, anticarcinogenic and antihypertensive properties (Lourens-Hattingh & Viljeon, 2001). Antihypertension has been reported to be mediated through inhibition of angiotensin-converting enzyme (ACE) (Nakamura et al., 1995). This enzyme plays a major role in the regulation of blood pressure. ACE converts angiotensin-I to a vasoconstrictor, angiotensin-II, and the inactivation of the vasodilator bradykinin. ACE inhibition results in an antihypertensive effect (Fuglsang, Rattray, Nilsson, & Nyborg, 2003; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). Many ACE-inhibitory peptides have been derived from food proteins (Donkor, Henriksson, Vasiljevic, & Shah, 2005; Saito et al., 2000; Wu & Ding, 2001).

The ability of lactic acid bacteria (LAB) to ferment the available carbohydrates in a growing medium varies with strains. Matsuoka, Sasago, and Sekiguchi (1968) found that *Streptococuss thermophilus* produced a greater amount of acid in soymilk than did *Lactococcus lactis* or *L. delbrueckii* ssp. *bulgaricus*. Mital, Steinkraus, and Naylor (1974) also reported that certain organisms, such as *S. thermophilus*, *L. acidophilus*, *L. cellobiosis* and *L. plantarum*, which utilize sucrose, exhibited significant growth and produced substantial amounts of acid in soymilk. Others, such as *L. delbrueckii* ssp. *bulgaricus*, grew poorly in soymilk because of their inability to ferment sucrose and other carbohydrates in soymilk. A similar finding was also reported by Wang, Kraidej, and Hesseltine (1974). The use of LAB in preparing fermented soy products has received much attention (Cheng, Thompson, & Brittin, 1990; Karleskind, Laye, Halpin, & Morr, 1991; Lee, Morr, & Seo, 1990). Several studies on α-galactosidase activity and metabolism of α-galactosyl oligosaccharides by *Bifidobacterium* strains in soymilk have been reported but there is a lack of detailed information in the literature about the behaviour of probiotic organisms (*L. acidophilus* and *L. casei*) and their importance as part of the starter cultures for making fermented soy products. The aims of this study were (a) to assess the suitability of soymilk as a substrate for growth and acid development by selected probiotic organisms and by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, (b) to examine the metabolism of oligosaccharides by these selected organisms, and (c) to monitor their proteolytic and ACE-inhibitory activities in soymilk.

2. Materials and methods

2.1. Bacterial cultures

Pure strains of *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. casei* LAFTI® L26 were kindly provided by DSM Food Specialties (Moorebank, NSW, Australia). *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb1466, *L. acidophilus* La4962, *B. longum* Bl 536 and *L. casei* Lc279 were obtained from the culture collection of Victoria University (Werribee, Australia). The lyophilized organisms were propagated in deMann Rogosa Sharpe (MRS) broth (Oxoid, West Heidelberg, Australia) according to the manufacturer's instructions at 37 °C with the exception of *L. delbrueckii* ssp. *bulgaricus* Lb1466 which was grown at 42 °C. For propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% (w/v) L-cysteine hydrochloride to provide anaerobic condition and to stimulate their growth (Ravula & Shah, 1998). After three successive transfers in MRS, the activated organisms were inoculated at 1% (v/v) level into 10 ml of sterilized commercial soymilk (Simply Soy, Sanitarium, NSW, Australia) supplemented with 2% (w/v) glucose and 1% (w/v) yeast extract for the manufacturing of the fermented soymilk.

2.2. Extraction of crude α-galactosidase

One of the requirements for good growth of cultures in soy-based media is the activity of α-galactosidase. All organisms (*L. acidophilus* L10, *B. lactis* B94, *L. casei* L26, *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb1466, *L. acidophilus* La4962, *B. longum* Bl536 and *L. casei* Lc279) were assessed for α-galactosidase activity according to the methods of Scalabrini et al. (1998) and Tsangalis and Shah (2004). Briefly, the organisms were activated by two successive propagations in MRS broth at 37 °C for 20 h. Subsequently, 5% (v/v) of active culture was inoculated into 250 ml of MRS broth and incubated at 37 °C for 48 h. In order to ascertain the metabolic characteristics of probiotics and *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in soymilk, α-galactosidase activity was examined in MRS basal broth supplemented with 2% (w/v) glucose, 2% (w/v) raffinose or a mixture of 1% (w/v) each of raffinose and glucose. During fermentation, 50 ml aliquots were withdrawn aseptically at 6, 12, 24, and 48 h and stored at 2 °C. Bacterial cells were harvested by centrifuging at 4000*g* for 10 min at 4 °C, using a Sorvall RT7 refrigerated centrifuge (Newtown, Conn., USA). The cell pellet was washed in 20 ml of cold 50 mM sodium citrate buffer (pH 5.5) and centrifuged at 4000*g* for 10 min and this was repeated twice. Finally cells were resuspended in 10 ml of the same buffer, placed in an ice bath and sonicated (Unisonics, Pty Ltd. Sydney, Australia) three times for 5 min. The cell debris was removed by centrifugation at 10,000*g* for 30 min at 4 °C. The supernatant was used as a crude enzyme extract.

2.3. Assay for α-galactosidase

Crude enzyme extracts from the organisms were assayed for α-galactosidase activity according to the method of Scalabrini et al. (1998). Briefly, 250 μl of crude enzyme extract were mixed with 500 μl of 5 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) and incubated at 37 °C for 30 min. The reaction was stopped by addition of 500 μl of cold 0.2 M sodium carbonate. The α-galactosidase activity was determined by the rate of hydrolysis of *p*NPG. The amount of *p*-nitrophenol released was measured with a spectrophotometer (LKB NOVASPEC II, Pharmacia, LKB Biochrom, England) at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from *p*NPG per millilitre per min under assay conditions. The specific activity was expressed as units (U) of α-galactosidase activity per milligramme of protein.

2.4. Determination of protein concentration

The protein concentration of the crude enzyme extracts was estimated as described previously (Donkor, Henriksson, Vasiljevic, & Shah, 2006). An aliquot (0.1 ml) of the crude enzyme and 3 ml of the Bradford reagent were vortexed gently to mix thoroughly and the samples were incubated at room temperature for 30 min, after which the absorbance was measured at 595 nm. A standard calibration curve was prepared using known concentrations of bovine serum albumin (Sigma). The protein concentration of all samples was determined from the standard curve.

2.5. Organic acids production

Analysis of organic acids was performed using the method of Shin, Lee, Pestka, and Ustunol (2000) with some modifications. Briefly, 3 ml of fermented soymilk were extracted and filtered into HPLC vials for organic acids determination by HPLC. Separation of organic acids was achieved using a Varian HPLC (Varian Analytical Instruments, CA, USA) fitted with an Aminex HPX–87 H, 300 × 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, CA, USA) and a guard column maintained at 65 °C. The mobile phase was 0.01 M H_2SO_4 and the flow rate was maintained at 0.6 ml/min. A UV/vis detector was used at 220 nm. Quantification of acetic and lactic acids was carried out using a standard curve, obtained from external standard solutions of pre-determined concentrations of acetic and lactic acids, as described previously (Donkor et al., 2005).

2.6. Performance of dairy cultures in soymilk

2.6.1. Fermentation of soymilk

Eight batches of 250 ml of commercial soymilk were sterilized (100 °C for 30 min) and aseptically inoculated with 1% (v/v) of each culture and incubated at 42 °C for 48 h. All fermentations were performed in triplicate. A control consisted of uninoculated soymilk. During fermentation, aliquots from each batch were taken at 0, 6, 12, 24, and 48 h to monitor cell growth, pH changes, organic acids production, metabolism of oligosaccharides, and proteolytic and ACE-inhibitory activities.

2.6.2. Cell growth

The cell growth of each organism was assessed by enumerating bacterial population during 48 h of fermentation in soymilk, as described previously (Donkor et al., 2005). Enumeration of the bacteria was performed on MRS agar (MRS growth medium, Difco, supplied by Amyl media, Dandenong, Australia). Anaerobic jars and gas generating kits (Anaerobic system BR38, Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Plates in duplicate were incubated anaerobically for 72 h at 37 °C for *L. acidophilus*, *L. casei*, and *Bifidobacterium* spp., for 72 h at 42 °C for *L. delbrueckii* ssp. *bulgaricus* and aerobically for 72 h at 37 °C for *S. thermophilus*. Plates containing 25–250 colonies were counted and recorded as colony-forming units (cfu) per gram of the fermented soymilk.

2.6.3. pH changes and production of organic acids

During culture growth, the main metabolic products are organic acids, particularly lactic and acetic acids. The pH changes in batches of soymilk were monitored during fermentation at 0, 6, 12, 24 and 48 h, using a pH meter (HANNA instruments 8417, Singapore). The concentration of organic acids was measured according to Donkor et al. (2005).

2.6.4. Determination of oligosaccharides

The extraction of sugars from fermented and unfermented soymilk samples was performed using the method described previously by Scalabrini et al. (1998) with some modifications. Briefly, 3 ml aliquots were centrifuged at 14,000*g* for 30 min for protein removal, followed by filtration using a 0.20 μm membrane filter (Schleicher & Schuell, Dassel, Germany). The concentrations of raffinose, stachyose and sucrose were determined with a Varian HPLC (Varian Analytical Instruments, CA, USA) fitted with an Alltima amino column (250 × 4.6 mm × 5 μm 100 Å) and corresponding guard column (Alltech Associates, Deerfield, IL, USA) maintained at 30 °C and an RI detector (ERC-7515 A, ERMA Cr. Inc., Kawaguchi City, Japan). The mobile phase consisted of 75% acetonitrile and 25% distilled water and was maintained at a flow rate of 1 ml/min, isocratically. A 20 μl injection volume was used for both samples and standards. The retention times of the standards for raffinose, stachyose and sucrose (Sigma) were 11.4, 19.1 and 7.8 min, respectively. Standard stock solutions of raffinose (2.53 g/100 ml), stachyose (2.03 g/100 ml) and sucrose (2.53 g/100 ml) were used for preparation of standard calibration curves. The concentration of oligosaccharides was derived from the standard curve and was expressed as milligrams of sugar per 100 ml of soymilk.

2.6.5. Proteolytic activity of cultures in fermented soymilk

Proteolysis during fermentation of soymilk was determined by measuring free NH_3 groups using the *o*-phthaldialdehyde (OPA) method (Church, Swaisgood, Porter, & Catignani, 1983) with some modifications, as described previously (Donkor et al., 2005). Briefly, 3 ml aliquots of fermented soymilk were mixed with 0.75% (w/v) trichloroacetic acid (TCA) and filtered using an Advantec 231 filter (M.F.S. Inc., USA). The filtrate was consequently mixed with 3 ml of OPA reagent and left at room temperature (~20 °C) for 2 min. The absorbance of the solution was measured by a spectrophotometer (LKB NOVASPEC II, Pharmacia, LKB Biochrom, England) at 340 nm. The relative proteolytic activity of these organisms was expressed as the absorbance of free amino groups, measured using the untreated soymilk as a blank. Triplicate aliquots from each TCA filtrate were analyzed.

2.6.6. In vitro inhibition of angiotensin-I-converting enzyme

One millilitre of 0.75% TCA was added to 3 ml of fermented soymilk and the mixture was centrifuged, filtered and the filtrate was assayed for ACE-I activity using the method of Cushman and Cheung (1971) with some modifications (Donkor et al., 2005). Briefly, the reaction mixture contained 200 μl of hippuryl–histidyl–leucine (Sigma) buffer, 60 μl of sodium borate buffer, 20 μl of sample extract and 20 μl of ACE solution. After 30 min of incubation at 37 °C, the reaction was stopped by addition of 250 μl of 1 M HCl. The hippuric acid formed by the action of ACE was extracted with ethyl acetate. The ethyl acetate was evaporated to dryness and the amount of hippuric acid was measured spectrophotometrically at 228 nm. The extent of inhibition was calculated as described earlier (Donkor et al., 2005).

2.7. Statistical analysis

All results obtained were analyzed as a split plot in time design using the general linear model (GLM) procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by fulfilling Huynh–Feldt (H–F) conditions (Littell, Henry, & Ammerman, 1998). Where appropriate, one-way ANOVA and correlational analysis were employed using Microsoft® Excel StatPro™ (Albright, Winston, & Zappe, 1999) and the multicomparison of means was assessed by Tukey's test. The statistical level of significance was preset at 0.05.

3. Results and discussion

3.1. α-Galactosidase specific activity of probiotic organisms and of L. delbrueckii ssp. bulgaricus and S. thermophilus

The α-galactosidase activities of the probiotic organisms and those of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are shown in Table 1. The organisms exhibited α-galactosidase activity at varying degrees, and fermentation time was a significant ($P = 0.0007$) factor for α-galactosidase activity during the 48 h incubation at 37 °C. This might be due to growth differences in the respective media which likely determined the amount of enzymes produced during fermentation. Interestingly, α-galactosidase activity in the medium containing raffinose declined substantially during 24–36 h of fermentation for some organisms but increased significantly ($P < 0.05$) for all microorganisms at 48 h. This followed the growth pattern of the organisms in the medium during the fermentation period. However, *B. lactis* B94 grew better than the other organisms in that medium as α-galactosidase activity increased significantly ($P < 0.05$) until the end of fermentation. This suggests that *B. lactis* B94 may have hydrolyzed raffinose, mainly by producing acetate and lactate from the bifidus carbohydrate catabolism pathway. Scalabrini et al. (1998) also reported the production of α-galactosidase by *Bifidobacterium* strains in a modified medium. On the other hand, *L. delbrueckii* ssp. *bulgaricus* showed the lowest α-galactosidase activity in the medium containing raffinose (compared to other organisms). Although α-galactosidase specific activity in the glucose/raffinose medium was not as high as that of medium containing raffinose, the trend of activity was similar. The presence of glucose in MRS medium, on the other hand, resulted in high α-galactosidase activity for the majority of the organisms in comparison to other media, except *B. lactis* B94, in 2% raffinose. All organisms grew well in MRS medium, which suggests that an increase in α-galactosidase is due to an increase in cell density. After reaching the log phase, α-galactosidase activity was reduced ($P < 0.05$). This reduction was most probably a result of depletion of the readily available carbon source (Table 1).

The presence of 1% each of glucose and raffinose did not stimulate ($P > 0.05$) the synthesis of α-galactosidase as much as did that containing 2% raffinose (Table 1). Tsangalis and Shah (2004) reported that 1% raffinose supplementation increased the production of α-galactosidase for *Bifidobacterium* (BB536 and BP20099) strains but had little or no effect on the α-galactosidase activity of *Bifidobacterium* (BB12 and BL1941). Since raffinose is an α-galactoside sugar found in soymilk, these selected organisms may grow well in soymilk for manufacture of soy-based yoghurt.

Table 2 shows the effects of raffinose and glucose on the production of organic acids by probiotic organisms and by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in supplemented media. *B. lactis* B94 exhibited the highest ($P < 0.05$) lactic and acetic acids production in 2% raffinose medium with significant ($P < 0.05$) increases during fermentation, as opposed to other organisms which produced organic acids in comparable quantities ($P > 0.05$). On the other hand, *B. longum* Bl536 produced low amounts of acetic acid in comparison to *B. lactis* B94. Scalabrini et al. (1998) reported that some *Bifidobacterium* strains produced relatively low quantities of acetic acid in a modified

Table 1
α-Galactosidase activity of probiotic and yoghurt cultures in supplemented media at 37 °C for 48 h

Strain	Time, h	α-Galactosidase specific activity*, U/mg		
		Raffinose	Glucose/raffinose	Glucose
L. delbruekii ssp. *bulgaricus* Lb1466	12	7.02^{a}	9.35^{a}	14.8^{a}
	24	5.18^{a}	7.69^{a}	12.7^{a}
	36	5.57^{a}	6.90^{ab}	14.4^{a}
	48	6.72^{a}	17.6^{bc}	14.6^{a}
S. thermophilus St1342	12	11.3^{a}	9.60^{a}	34.1^{a}
	24	9.68^{a}	6.03^{b}	13.8^{b}
	36	7.40^{a}	2.79^{c}	13.9^{b}
	48	15.1^{ab}	13.1^{d}	11.3^{b}
L. acidophilus L10	12	10.2^{Aa}	9.22^{Aa}	18.0^{Aa}
	24	9.12^{Aa}	3.49^{Cb}	13.0^{Cb}
	36	10.2^{Aa}	4.28^{Db}	11.8^{Db}
	48	14.9^{ABa}	9.87^{Ea}	11.9^{Eb}
L. acidophilus La4962	12	9.38^{Aa}	7.23^{Ba}	42.1^{Ba}
	24	7.78^{Aa}	4.70^{BCDb}	21.4^{Bb}
	36	4.02^{CAa}	5.46^{BDab}	10.8^{BCDEc}
	48	18.9^{Bb}	8.91^{EAac}	28.9^{Bd}
B. lactis B94	12	11.1^{Aa}	7.64^{Aa}	41.9^{Aa}
	24	25.1^{Aa}	4.57^{Ca}	34.3^{Cb}
	36	155^{Ab}	3.94^{Da}	24.5^{Bbc}
	48	183^{Cb}	11.1^{Eab}	14.5^{Ebcd}
B. longum Bl536	12	14.6^{ABab}	22.1^{Ba}	23.3^{Ba}
	24	10.7^{ABa}	9.13^{AEb}	28.4^{Ba}
	36	8.68^{ABa}	10.0^{AEbc}	12.3^{BDEb}
	48	19.1^{ABa}	9.74^{AEbcd}	21.0^{Bac}
L. casei L26	12	16.7^{Aa}	9.21^{Aa}	18.0^{Aa}
	24	8.19^{ABb}	10.8^{Aa}	35.7^{Bb}
	36	15.1^{Ca}	5.98^{Cb}	7.70^{Cabc}
	48	18.9^{Da}	13.8^{Db}	9.97^{Ea}
L. casei Lc279	12	6.02^{Ba}	9.21^{Aa}	27.4^{Ba}
	24	8.42^{Ba}	3.78^{Bb}	30.7^{Ba}
	36	6.81^{Ba}	5.92^{BCbc}	22.3^{Aa}
	48	14.0^{CAa}	9.82^{Aa}	10.8^{ACEb}
SEM		6.81		

^{abcd} Means in the same column for a particular strain with different small letter superscripts are significantly different.
^{ABCDE} Means in the same column for particular strains with different capital letter superscripts are significantly different.
* One unit of α-galactosidase activity was defined as the amount of enzyme required to release 1 nmol of *p*-ntrophenol from *p*-nitrophenyl-α-D-galactopyranoside/ml/min under assay conditions. Results presented as a mean of three observations; SEM, pooled standard error of the mean (0.03); the level of significance was preset at $P = 0.05$.

medium. The production of acetic acid in 1% raffinose and 1% glucose by *L. acidophilus* (La4962 and L10), *B. lactis* B94 and *B. longum* Bl536, *L. casei* Lc279 and *L. casei* L26, *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 showed a slight ($P > 0.05$) variation in concentrations compared to that produced in 2% raffinose. The mixture of glucose/raffinose stimulated higher ($P < 0.05$) production of lactic acid by all organisms studied than did the medium containing 2% raffinose. The MRS broth, which contained 2% glucose, showed similar production of lactic acid to that of the mixture but low ($P < 0.05$) production of acetic acid for all the organisms (Table 2). Our study showed that metabolism of raffinose as the sole energy source produced substantially more acetic acid than lactic acid.

3.2. Sugars metabolism in soymilk

Tables 3 and 4 show the utilization of raffinose, stachyose and sucrose and the production of lactic and acetic acids. The levels of breakdown of raffinose and stachyose in soymilk varied and appeared to depend on the α-galactosidase activity of the organism (Table 1). *S. thermophilus* St1342, *L. acidophilus* La4962 and *B. lactis* B94 reduced raffinose significantly ($P < 0.05$) by 64.5%, 55.9% and 77.4%, respectively, whereas the remaining organisms showed less than 30% reduction after 48 h. Scalabrini et al. (1998) found that *Bifidobacterium* strains metabolized raffinose in soymilk, as opposed to yoghurt cultures, which did not reduce raffinose and stachyose during growth in soymilk. Our study was in line with that

Table 2
Production of organic acids by selected probiotic and yoghurt cultures in supplemented MRS at 37 °C for 48 h

Strain	Time, h	Organic acids, mg/ml					
		Raffinose		Glucose/Raffinose		Glucose	
		LA[a]	AA[b]	LA	AA	LA	AA
L. delbruekii ssp. *bulgaricus* Lb1466	12	1.77^{a}	4.28^{a}	9.89^{a}	4.46^{a}	8.47^{a}	2.27^{a}
	24	1.51^{a}	5.76^{ba}	9.96^{a}	4.04^{a}	12.1^{b}	2.24^{a}
	36	1.67^{a}	5.60^{ab}	11.4^{a}	5.03^{a}	13.2^{b}	2.23^{a}
	48	1.57^{a}	5.87^{b}	10.5^{a}	4.51^{a}	13.7^{b}	2.34^{a}
S. thermophilus	12	1.66^{a}	4.90^{a}	10.4^{a}	4.56^{a}	8.42^{a}	2.19^{a}
	24	1.64^{a}	5.78^{a}	11.4^{a}	6.49^{b}	11.2^{b}	2.28^{a}
	36	1.67^{a}	5.75^{a}	10.5^{a}	5.66^{ab}	13.2^{b}	2.22^{a}
	48	1.69^{a}	6.15^{a}	10.6^{a}	3.79^{a}	13.5^{b}	2.23^{a}
L. acidophilus	12	1.40^{Aa}	4.08^{Aa}	11.8^{Aa}	6.62^{Aa}	9.93^{Aa}	2.45^{Aa}
	24	1.03^{Ba}	4.32^{BAa}	11.8^{Aa}	7.54^{Ba}	13.5^{BCb}	2.51^{Aa}
	36	1.36^{Aa}	5.04^{Ba}	10.4^{Aa}	6.44^{Aa}	13.6^{BDb}	2.69^{Aa}
	48	1.11^{Aa}	4.50^{Ba}	10.4^{Aa}	5.92^{Aa}	13.6^{BEb}	2.81^{Ca}
L. acidophilus La	12	1.57^{ACa}	5.86^{Ba}	10.3^{Aa}	4.52^{ACa}	9.31^{Aa}	2.26^{Aa}
	24	1.48^{Aa}	5.77^{Ba}	11.5^{Aa}	6.39^{Aa}	13.0^{Bb}	2.26^{Aa}
	36	1.53^{Aa}	5.69^{Ba}	11.2^{Aa}	6.77^{Aa}	13.3^{Bb}	1.93^{Ba}
	48	1.51^{Aa}	5.66^{Ba}	10.3^{Aa}	4.62^{ABCa}	13.3^{Bb}	2.26^{Aa}
B. lactis B94	12	1.54^{Aa}	6.14^{Aa}	10.4^{Aa}	4.86^{Aa}	9.20^{Aa}	2.46^{Aa}
	24	2.16^{Aa}	5.96^{Aa}	11.2^{Aa}	6.61^{Aa}	13.0^{BCb}	2.28^{Aa}
	36	5.02^{Bab}	8.07^{Aab}	11.3^{Aa}	6.52^{Aa}	13.6^{BDb}	2.30^{Aa}
	48	6.34^{Db}	12.84^{Bb}	9.36^{Aa}	3.65^{ABab}	13.1^{BEb}	2.30^{Aa}
B. longum Bl536	12	2.62^{ABa}	3.92^{ACa}	10.3^{Aa}	4.69^{ABa}	9.31^{Aa}	2.20^{Aa}
	24	1.24^{ACa}	5.92^{ACa}	11.4^{Aa}	5.74^{ABa}	12.9^{Bb}	2.28^{Aa}
	36	1.70^{ACa}	5.40^{ACa}	11.32^{Aa}	6.79^{ACa}	13.5^{Bb}	2.32^{Aa}
	48	1.25^{ACa}	5.69^{ACa}	10.4^{Aa}	4.48^{ABa}	13.5^{Bb}	2.35^{Aa}
L. casei L26	12	1.41^{Aa}	5.24^{Aa}	10.6^{Aa}	4.98^{Aa}	8.10^{Aa}	2.24^{Aa}
	24	1.12^{Aa}	5.25^{Aa}	12.2^{Aa}	7.07^{Bab}	11.7^{BCb}	2.29^{Aa}
	36	1.39^{Aa}	5.32^{Aa}	11.1^{Aa}	4.07^{ACabc}	13.6^{BDb}	2.30^{Aa}
	48	1.21^{Aa}	5.35^{Aa}	12.1^{Aa}	5.52^{ACab}	13.5^{BEb}	2.25^{Aa}
L. casei Lc279	12	1.57^{Aa}	5.51^{Aa}	9.40^{Aa}	3.53^{ACa}	9.02^{Aa}	2.08^{Aa}
	24	1.32^{Aa}	5.70^{Aa}	9.83^{Aa}	3.53^{ACa}	13.4^{Bb}	2.29^{Aa}
	36	1.62^{Aa}	5.79^{Aa}	9.81^{Aa}	4.12^{ACa}	13.6^{Bb}	2.34^{Aa}
	48	1.19^{Aa}	4.65^{Aa}	10.3^{Aa}	4.56^{ACa}	13.7^{Bb}	2.30^{Aa}
SEM		0.49	0.35	0.49	0.35	0.49	0.35

[abc] Means in the same column for a particular strain with different small letter superscripts are significantly different.
[ABCDE] Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM, pooled standard error of the mean (0.49 and 0.35).
[a] LA, lactic acid.
[b] AA, acetic acid.

reported by Scalabrini et al. (1998), which showed that raffinose, was substantially metabolized by *Bifidobacterium* strains. The organisms, in general, metabolized stachyose by over 40% after 48 h, with *B. lactis* B94 and *L. acidophilus* L10 showing the highest hydrolyses of 63.5% and 57.5%, respectively. Our findings are in line with those of Mital and Steinkraus (1975), who reported that fermentation of soymilk with lactic cultures possessing α-galactosidase activity reduced raffinose and stachyose contents. Overall, the sucrose concentration in soymilk was significantly ($P < 0.05$) reduced by *S. thermophilus* St1342, *B. lactis* B94 and *L. casei* Lc279 after 48 h (Table 3).

3.3. Proteolytic activity

The proteolytic activity of the organisms in soymilk is presented in Fig. 1. The amount of liberated amino groups and peptides increased only slightly ($P > 0.05$) during fermentation from 0 to 12 h for *L. acidophilus* La4962, *B. longum* Bl536, *B. lactis* B94, *L. casei* Lc279 and *S. thermophilus* St1342, but increased significantly ($P < 0.05$) for the majority of the strains from 24 to 48 h (Fig. 1). *L. delbrueckii* ssp. *bulgaricus* Lb1466 showed the highest proteolytic activity ($P < 0.005$) (in comparison to other organisms). *L. delbrueckii* ssp. *bulgaricus* has been considered as highly proteolytic in dairy systems (Abraham,

Table 3
Changes in concentrations of raffinose, stachyose and sucrose in soymilk during fermentation with probiotic organisms and yoghurt culture for 0, 6, 12, 24, and 48 h at 42 °C

Sugar/strain	Sugar concentration, mg /100 ml Time, h				
	0	6	12	24	48
Raffinose					
Lb1466	3.49^{a}	3.07^{a}	2.91^{a}	2.78^{a}	2.69^{Aa}
St1342	3.49^{a}	2.73^{a}	2.55^{a}	2.38^{a}	1.24^{Aa}
L10	3.49^{a}	3.33^{Aa}	3.21^{Aa}	3.20^{Aa}	2.51^{Aa}
La4962	3.49^{a}	2.51^{Aa}	2.23^{Ba}	1.81^{Ba}	1.54^{Aa}
B94	3.49^{a}	2.50^{Aab}	2.19^{Aab}	1.21^{Ab}	0.79^{Ab}
Bl536	3.49^{a}	3.43^{Ba}	3.15^{Ba}	2.76^{Ba}	2.63^{Ba}
L26	3.49^{a}	3.45^{Aa}	2.95^{Aa}	2.87^{Aa}	2.82^{Aa}
Lc279	3.49^{a}	3.43^{Aa}	3.21^{Aa}	2.59^{Aa}	2.71^{Aa}
SEM			0.52		
Stachyose					
Lb1466	14.1^{a}	8.99^{a}	8.64^{a}	8.48^{a}	7.90^{Aa}
St1342	14.1^{a}	13.8^{a}	10.74^{a}	10.2^{a}	8.38^{Aa}
L10	14.1^{a}	10.3^{Aa}	9.7^{Aa}	7.40^{Aa}	6.00^{Aa}
La4962	14.1^{a}	12.1^{Ba}	11.0^{Ba}	10.07^{Ba}	8.43^{Aa}
B94	14.1^{a}	11.2^{Aab}	9.81^{Aab}	7.85^{Aab}	5.16^{Ab}
Bl536	14.1^{a}	13.9^{Ba}	12.8^{Ba}	12.8^{Ba}	11.11^{Ba}
L26	14.1^{a}	13.0^{Aa}	11.0^{Aa}	9.62^{Aa}	8.22^{Aa}
Lc279	14.1^{a}	12.2^{Ba}	10.9^{Aa}	9.22^{Ab}	9.20^{Bb}
SEM			3.23		
Sucrose					
Lb1466	202^{a}	188^{a}	177^{a}	132^{a}	123^{Aa}
St1342	202^{a}	201^{ab}	164^{ab}	158^{ab}	87.7^{Bb}
L10	202^{a}	197^{Aa}	194^{Aa}	136^{Aa}	116^{Aa}
La4962	202^{a}	175^{Ba}	168^{Ba}	160^{Ba}	149^{Ba}
B94	202^{a}	164^{Aab}	161^{Aab}	124^{Aab}	83.5^{Ab}
Bl536	202^{a}	196^{Bab}	142^{Bab}	138^{Bab}	113^{Bb}
L26	202^{a}	176^{Aa}	116^{Aa}	110^{Aa}	103^{Aa}
Lc279	202^{a}	161^{Bb}	139^{Bc}	109^{Ad}	77.0^{Be}
SEM			15.21		

Lb1466, *L. delbruekii* ssp. *bulgaricus*; St1342, *S. thermophilus*; *L*10 & La4962, *L. acidophilus*; B94, *B. lactis*; Bl536, *B. longum*; L26, *L. casei*; Lc279, *L. casei*.
^{abcde} Means in the same column for a particular strain with different small letter superscripts are significantly different.
^{AB} Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM, pooled standard error of the mean (0.52, 3.23 and 15.21).

DeAntoni, & Anon, 1993; Shihata & Shah, 2000). Our study showed that *L. delbrueckii* ssp. *bulgaricus* strains are also proteolytic in soymilk. *B. lactis* B94 was the next highest proteolytic organism in soymilk showing a significant ($P < 0.005$) production of free amino groups during 24–48 h of fermentation. The rest of the organisms exhibited varying degrees of proteolytic activity and all showed increasing proteolysis ($P < 0.005$) with time (Fig. 1). The proteolytic activity in soymilk appeared to be strain-specific ($P < 0.0001$) and also was time-dependent ($P < 0.0001$). Other studies have also reported proteolytic activities with LAB in soy-based products (Donkor et al., 2005; Omafuvbe, Abiose, & Shonukan, 2002). Strain specificity of proteolytic activity (Fuglsang et al., 2003; Shihata & Shah, 2000) is not confined to a milk medium only but is also exhibited in soymilk, as was observed in our study.

Some amino acids and peptides were used by the organisms for cell growth and survival (Nielsen, Petersen, & Dambmann, 2001). Therefore high proteolytic activity of these organisms in soymilk may have contributed to appreciable cell growth, in addition to their ability to metabolize stachyose and raffinose as energy sources. The correlation between proteolytic activity and growth, which ranged from 0.61 for *S. thermophilus* St1342 to 0.98 for *L. casei* Lc279, further indicates the substantial effect of proteolytic activity on bacterial growth. The influence of proteolytic activity on cell growth of *Bifidobacterium* sp. and some selected probiotic organisms as

Table 4
Metabolic activity of investigated cultures during growth in soymilk for 48 h at 42 °C

Strain	Time, h	pH	Cell counts, cfu/ml	Organic acids, mg/ml	
				Lactic	Acetic
L. delbrueckii ssp. *bulgaricus* Lb1466	0	6.67^{a}	6.69^{a}	0.00^{a}	0.00^{a}
	6	6.53^{b}	7.21^{a}	0.01^{a}	0.00^{a}
	12	6.45^{b}	7.43^{ab}	0.01^{ab}	0.02^{a}
	24	5.82^{bc}	7.82^{b}	0.03^{b}	0.06^{b}
	48	5.59^{abcd}	7.88^{b}	0.04^{b}	0.07^{b}
S. thermophilus St1342	0	6.66^{a}	7.44^{a}	0.01^{a}	0.00^{a}
	6	6.50^{b}	7.59^{a}	0.01^{a}	0.02^{b}
	12	6.39^{c}	8.04^{b}	0.02^{b}	0.06^{bc}
	24	4.90^{d}	8.23^{b}	0.17^{bc}	0.07^{bcd}
	48	4.00^{e}	8.24^{b}	0.33^{bcd}	0.07^{bcd}
L. acidophilus L10	0	6.64^{Aa}	6.66^{Aa}	0.01^{Aa}	0.00^{Aa}
	6	6.46^{Aab}	6.94^{Cab}	0.01^{Aa}	0.00^{Aa}
	12	6.16^{Bb}	7.30^{Bb}	0.02^{Aa}	0.00^{Aa}
	24	4.93^{Dbc}	7.30^{Bb}	0.09^{Aa}	0.01^{Aa}
	48	4.10^{Ebcd}	7.37^{Bb}	0.26^{Bb}	0.03^{Aa}
L. acidophilus La4962	0	6.66^{ACa}	7.67^{Ba}	0.02^{Aa}	0.00^{Aa}
	6	6.50^{ABa}	7.71^{Ba}	0.01^{Aa}	0.03^{ABb}
	12	6.44^{ABa}	8.08^{BDab}	0.02^{Aa}	0.05^{Bb}
	24	6.41^{ABa}	8.17^{BDb}	0.04^{Aa}	0.06^{Bbc}
	48	4.14^{BCEb}	8.81^{BDbc}	0.28^{Bb}	0.07^{Bbc}
B. lactis B94	0	6.62^{Aa}	7.25^{Aa}	0.00^{Aa}	0.00^{Aa}
	6	6.45^{Aa}	7.50^{Aab}	0.01^{Aa}	0.02^{Aa}
	12	6.43^{Aa}	7.86^{ABab}	0.02^{Aa}	0.05^{Bb}
	24	4.89^{Cb}	7.96^{ABCab}	0.09^{Aab}	0.11^{Cbc}
	48	4.42^{Cb}	8.44^{Eb}	0.19^{Cb}	0.12^{Cbc}
B. longum Bl536	0	6.60^{Aa}	7.24^{Aa}	0.01^{Aa}	0.01^{Aa}
	6	6.40^{Aa}	8.03^{AEab}	0.02^{Aa}	0.02^{ABa}
	12	6.22^{Aa}	8.82^{BEbc}	0.03^{Aa}	0.05^{Bba}
	24	4.55^{CBb}	8.84^{BCEbc}	0.21^{ABCb}	0.06^{Bba}
	48	3.81^{Bbc}	9.54^{BCDbc}	0.40^{Bbc}	0.06^{Bba}
L. casei L26	0	6.62^{Aa}	7.49^{Aa}	0.01^{Aa}	0.00^{Aa}
	6	6.36^{Aa}	8.81^{Cb}	0.02^{Aa}	0.03^{Cbc}
	12	6.13^{Cab}	8.26^{Dbc}	0.04^{Aa}	0.06^{Db}
	24	4.12^{Ebc}	8.79^{Ebd}	0.29^{Bb}	0.06^{Db}
	48	3.84^{Ebc}	9.13^{Fbd}	0.37^{Bbc}	0.05^{Db}
L. casei Lc279	0	6.62^{ADa}	7.29^{Aa}	0.01^{Aa}	0.01^{Aa}
	6	6.41^{ACa}	7.68^{Aab}	0.01^{Aa}	0.02^{BCbc}
	12	6.38^{ACa}	7.94^{BDbc}	0.02^{Aa}	0.05^{BDb}
	24	6.39^{ACa}	8.16^{BDbcd}	0.03^{Aa}	0.06^{BDb}
	48	3.97^{BDEb}	8.88^{BCEFbc}	0.33^{Bb}	0.05^{BDb}
SEM		0.05	0.10	0.01	0.00

^{abcde} Means in the same column for a particular strain with different small letter superscripts are significantly different.
^{ABCDEF} Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM, pooled standard error of the mean (0.05 and 0.10).

well as *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in soymilk was also reported by Donkor et al. (2005) and Kamaly (1997).

3.4. Viability and organic acids production in fermented soymilk

In general, probiotic cultures achieved the desired therapeutic level (10^8 cfu/ml) during growth by each organism in soymilk after 48 h of fermentation, as shown in Table 4. Organic acid production, pH decline and other metabolic activities occurred during the first 12–24 h of incubation, which corresponded to the exponential phase of the growth. *B. longum* Bl536 and *L. casei* L26 exhibited better growth ($P < 0.05$) throughout the cultivation than did other microorganisms. On the other hand, *L. acidophilus* L10 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 showed slow growth, one log cycle lower ($P < 0.05$) than did the rest of the organisms throughout the incubation (Table 4). *S. thermophilus* St1342 grew better than *L. delbrueckii* ssp. *bulgaricus* Lb1466 and produced more ($P < 0.05$) organic acids than did the latter during fermentation. Similarly, Wang

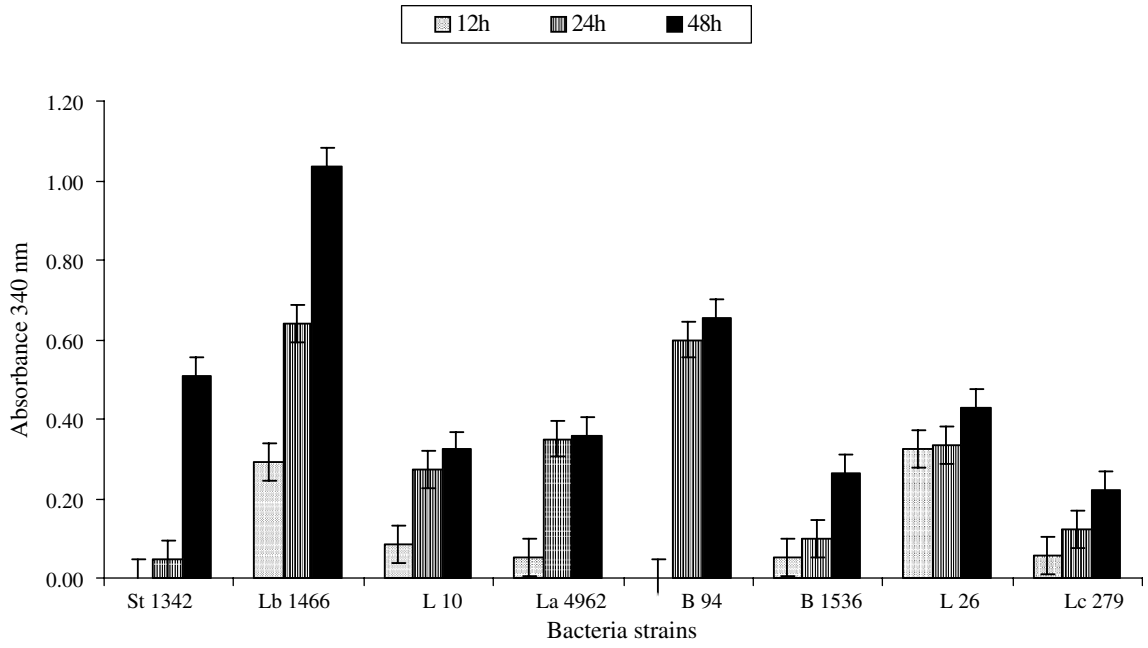

Fig. 1. The extent of proteolysis in soymilk fermented with probiotic strains (*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26, *L. acidophilus* La4962, *B. longum* Bl536 and *L. casei* Lc279) and yoghurt culture (*S. thermophilus* St1342, *L. delbrueckii* subsp. *bulgaricus* Lb1466) for 12, 24, and 48 h at 42 °C. (Error bars represent a pooled standard error of the mean; SEM = 0.14 absorbance units).

et al. (1974) reported that *L. delbrueckii* ssp. *bulgaricus* grew poorly in soymilk because of its inability to ferment sucrose and other soy carbohydrates. On the other hand, it has been demonstrated that *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 grew well as a mixed culture in soy yoghurt (Donkor et al., 2005). This was evident by the low ($P < 0.05$) α-galactosidase activity of *L. delbrueckii* ssp. *bulgaricus* Lb1466 (Table 1). Mital et al. (1974) also reported that certain organisms, such as *S. thermophilus*, *L. acidophilus, L. cellobiosis* and *L. plantarum*, which utilized sucrose, grew well and produced large amounts of acid in soymilk. However contrary to our finding, the selected organisms used in this study produced lower amounts of organic acids in soymilk even though they grew well. Liu (1997) also reported that LAB grew well in soymilk but produce less organic acids. The low levels of organic acid concentrations in fermenting soymilk presumably encouraged cell growth as noted in other studies (Angeles & Marth, 1971; Donkor et al., 2005; Kamaly, 1997; Liu, 1997). Furthermore, these selected strains possessed α-galactosidase (Table 1) and can utilize sucrose and other soy carbohydrates in soymilk as sources of energy, which enhanced better cell growth during 48 h of fermentation at 42 °C (Mital et al., 1974; Liu, 1997). Although the organisms in our study showed a consistent increase in cell concentration from the start of incubation until 24 h, the required pH of 4.5 was not reached within this time frame in comparison to the MRS medium with the exception of *L. casei* L26, that showed a pH decline of 4.12 (Table 4). However, after a prolonged incubation for 48 h, the pH of all batches declined to below 4.5. In general, the production of organic acids by all organisms in MRS was significantly higher ($P < 0.05$) than in those produced in the fermented soymilk from 0 to 48 h (Tables 2 and 4), thus making soymilk a potential medium for bacterial growth. Soymilk was reported previously as an appropriate growth medium for some lactic acid bacteria (Angeles & Marth, 1971; Donkor et al., 2005; Kamaly, 1997; Liu, 1997).

3.5. ACE-inhibitory activity

The ACE-inhibitory activity of all organisms studied is shown in Fig. 2. The percentage inhibition values between the bacterial strains varied ($P < 0.05$), indicating possible differences in ACE-inhibitory peptides produced by the organisms. In general, ACE-inhibitory activity appeared to be highest at 24 h for all organisms but the overall inhibition was below 50%. *B. longum* Bl536 showed highest activity (43.4%) at 48 h (as opposed to other organisms). *L. acidophilus* strains were not as proteolytic as were *L. delbrueckii* ssp. *bulgaricus* Lb1466, *B. lactis* B94 or *L. casei* L26 but they showed appreciable ACE-inhibitory activities. Similarly, Donkor et al. (2005) showed that LAB produced peptides, which exhibited *in vitro* ACE-inhibitory activity in soy yoghurt. Several studies have shown that soy protein hydrolysates, obtained mainly by alkaline hydrolysis, have lowered high blood pressure in hypertensive rats (Wu & Ding, 2002; Yang, Yang, Chen, Tzeng, & Han, 2004). The results showed that the production of ACE-inhibitory activity is strain-specific, however, not all peptides in fermented soymilk released as a result of proteolysis may express *in vitro* ACE-inhibition.

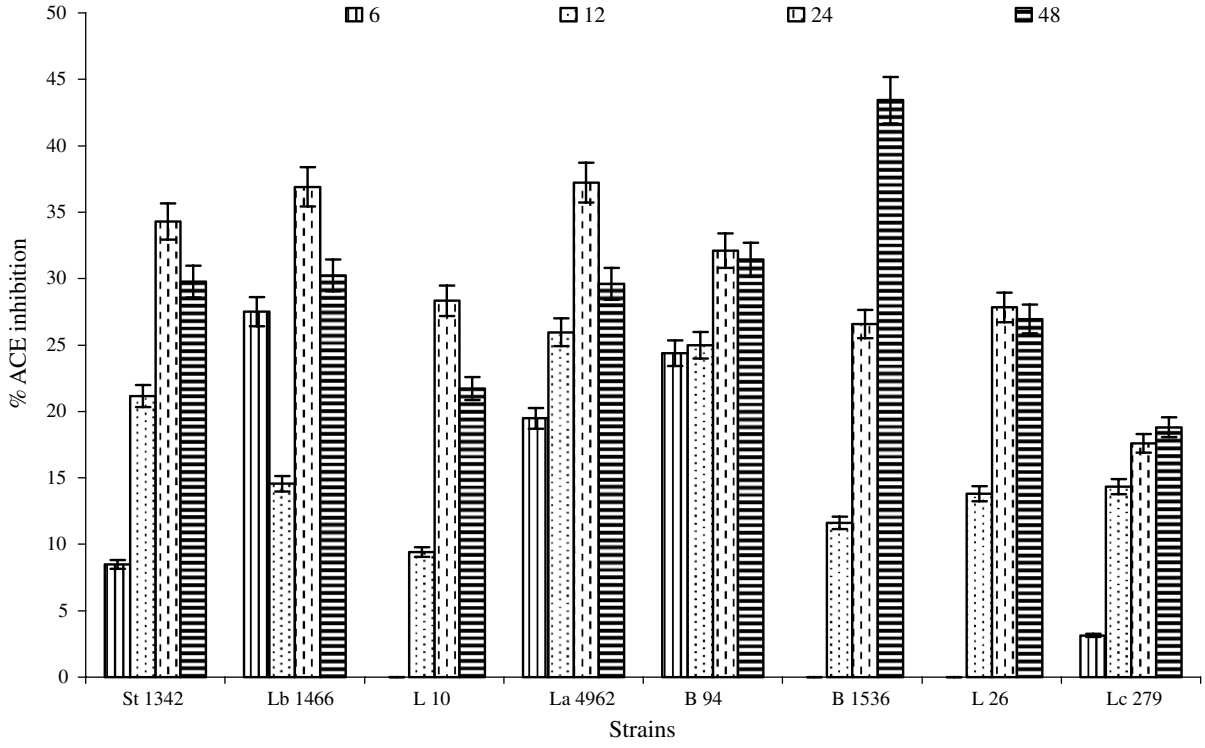

Fig. 2. Angiotensin-I-converting enzyme (ACE) inhibitory activities of fermented soymilk extracts obtained from individual culture (*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26, *L. acidophilus* La4962, *B. longum* Bl536, *L. casei* Lc279, *S. thermophilus* St1342, and *L. delbrueckii* subsp. *bulgaricus* Lb1466) fermentations in soymilk after 6, 12, 24 and 48 h at 42 °C. (Error bars represent a pooled standard error of the mean; SEM = 3.87).

4. Conclusions

Lactobacillus acidophilus (La4962 and L10), *B. lactis* B94 and *B. longum* Bl536, *L. casei* Lc279 and *L. casei* L26, *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 exhibited variable α-galactosidase activities with *B. lactis* B94 showing the highest activity in MRS-supplemented media. However, all organisms reached the desired therapeutic level (10^8 cfu/ml), likely due to their ability to metabolize oligosaccharides during fermentation in soymilk at 42 °C. The oligosaccharide metabolism depended on α-galactosidase activities. *B. lactis* B94, *S. thermophilus* St1342 and *L. acidophilus* La4962 reduced raffinose by 77.4%, 64.5% and 55.9%, respectively in soymilk. The organisms exhibited varying proteolytic activities and all showed increasing proteolysis with time. As a result of proteolytic activity, the peptides released showed ACE-inhibitory activity, which appeared to depend on strain. Thus, fermented soymilk could be converted into a rich functional product containing probiotics and bioactive compounds.

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