

Production of β -Glucosidase and Hydrolysis of Isoflavone Phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in Soymilk

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ABSTRACT: The study determined β -glucosidase activity of commercial probiotic organisms for hydrolysis of isoflavone to aglycones in fermenting soymilk. Soymilk made with soy protein isolate (SPI) was fermented with *Lactobacillus acidophilus* LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94, and *Lactobacillus casei* LAFTI® L26 at 37 °C for 48 h and the fermented soymilk was stored for 28 d at 4 °C. β -Glucosidase activity of organisms was determined using p -nitrophenyl β -D-glucopyranoside as a substrate and the hydrolysis of isoflavone glycosides to aglycones by these organisms was carried out. The highest level of growth occurred at 12 h for *L. casei* L26, 24 h for *B. lactis* B94, and 36 h for *L. acidophilus* L10 during fermentation in soymilk. Survival after storage at 4 °C for 28 d was 20%, 15%, and 11% greater ($P < 0.05$) than initial cell counts, respectively. All the bacteria produced β -glucosidase, which hydrolyzed isoflavone β -glycosides to isoflavone aglycones. The decrease in the concentration of β -glycosides and the increase in the concentration of aglycones were significant ($P < 0.05$) in the fermented soymilk. Increased isoflavone aglycone content in fermented soymilk is likely to improve the biological functionality of soymilk.

Keywords: aglycone, β -glucosidase, isoflavones, soy protein isolate, viability

Introduction

In addition to serving as a delivery medium for probiotic organisms to the consumer, soymilk has several nutritional advantages over dairy, milk including reduced level of cholesterol and saturated fat as well as the absence of lactose. Furthermore, soy products contain the isoflavone phytoestrogens with potential anticarcinogenic activity (Uzzan and Labuza 2004). Their antioxidant ability may also prevent oxidative damage in living tissue (Wei and others 1995; Ruiz-Larrea and others 1997). Isoflavones occur naturally in plants, in particular soybeans. Isoflavones are a part of the diphenol compounds, which are structurally and functionally similar to the human estrogen, estradiol, but are much less potent than estradiol (Tsangalis and others 2002). Because of this similarity, isoflavones were suggested to have preventive effects for many kinds of hormone-dependent diseases (Uzzan and Labuza 2004). Isoflavones in soy proteins in most soy foods are conjugated with sugars. The β -glucoside forms are not absorbed and require hydrolysis for bioavailability and subsequent metabolism. Hydrolysis occurs along the entire length of the intestinal tract by the action of both the brush border membrane- and the bacterial β -glucosidases (Setchell and others 2002). The aglycones are released and further metabolism of daidzein and genistein takes place. Intestinal biotransformations include dehydroxylation, reduction, C-ring cleavage, and demethylation. These reactions take place distally and presumably in the colon. However, the types of intestinal bacteria involved in isoflavone conversion to bioactive forms and the effectiveness of this microbial biotransformation are not well known.

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Bifidobacterium and *Lactobacillus* are the predominant members of the intestinal microflora; however, *Bifidobacterium* strains are widely studied for the production of β -glucosidase (Tochikura and others 1986; Tsangalis and others 2002; Hsu and others 2005) leading to transformation of isoflavones to bioavailable and bioactive forms (Tsangalis and others 2002, 2004; Wei and others 2007). However, production of β -glucosidase by other groups of bacteria has not been studied. Therefore, it will be interesting to study other groups of bacteria for the production of β -glucosidase using commercial probiotic organisms (*L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26) and biotransformation of isoflavone aglycones by these microorganisms in soymilk. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002).

The aims of this study were to evaluate β -glucosidase activity of selected probiotic organisms (*L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26) for hydrolysis of isoflavone to aglycones in soymilk fermented at 37 °C for 48 h, and to assess cell population and organic acid production during fermentation of soymilk at 37 °C for 48 h and storage at 4 °C for 28 d.

Materials and Methods

Propagation of cultures

Lactobacillus acidophilus LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94, and *L. casei* LAFTI® L26 were provided by DSM Food Specialties (Moorebank, NSW, Australia). These microorganisms have been reported to have probiotic properties (Crittenden and others 2005). Each culture was stored at -80 °C. Sterile 10-mL aliquots of MRS were inoculated with 1% of each culture and incubated at 37 °C for 20 h. For propagation of *Bifidobacterium*,

sterile MRS broth was supplemented with 0.05% L-cystein HCl to provide anaerobic conditions and stimulate growth (Ravula and Shah 1998). After 3 successive transfers in MRS broth, the activated organisms were transferred at 5% v/v to 10-mL aliquots of sterile soymilk supplemented with 2% glucose and 1% yeast extract. After a 2nd transfer in sterile soymilk without yeast extract the cultures were ready for the production of fermented soymilk.

Soymilk manufacture

Soymilk was prepared as per the method of Tsangalis and others (2004) by mixing 4% soy protein isolate (SPI) SUPRO[®] 590 IP (proximate composition: moisture 6.0%, protein, dry basis 90.0%, fat 1.5%, ash 5.0%, carbohydrate \leq 1.0%), supplied by SOLAE Co. (Chatswood, NSW, Australia) (w/v) in deionized water. For reconstitution, deionized water was heated to 40 °C prior to addition of SPI powder, followed by heating the mixture to 50 °C with constant stirring for 30 min to dissolve solid particles. A 11.4 L batch of soymilk was prepared and dispensed into 19 glass bottles containing 300 mL each. The bottles containing soymilk were sterilized by autoclaving at 121 °C for 15 min.

Fermentation of soymilk with microorganisms

Two sets of 6 glass bottles each containing 300 mL sterile soymilk were aseptically inoculated with *L. acidophilus* L10 at 5% (v/v) and incubated at 37 °C for 48 h. The bottles were labeled 0, 6, 12, 24, 36, and 48 h in order to facilitate withdrawing of aliquots at 0, 6, 12, 24, 36, and 48 h of fermentation. Aliquots of 50 mL from each of the 6 bottles were taken at 0, 6, 12, 24, 36, and 48 h of incubation. Each aliquot was divided into 20- and 30-mL portions in sterile 50-mL screw top falcon tubes for determination of β -glucosidase activity and pH and for enumeration of cell counts, whereas the 30-mL portions were immediately stored at -80 °C and later freeze dried using a Dynavac[®] FD 300 freeze drier (Rowville, Vic., Australia) for the analysis of isoflavones. The 2nd set of 6 bottles of fermented soymilk was stored at 4 °C for 28 d for determination of β -glucosidase activity and pH and for enumeration of bacteria. The experiments were repeated for *B. lactis* B94 and *L. casei* L26.

β -Glucosidase activity in soymilk

β -Glucosidase activity of the 3 microorganisms was determined in soymilk during fermentation at 37 °C for 6, 12, 24, 36, and 48 h. Five milliliter aliquot samples were taken at 0, 6, 12, 24, 36, and 48 h and the enzyme activity was determined immediately. The β -glucosidase activity was determined by measuring the rate of hydrolysis of p -nitrophenyl β -D-glucopyranoside (p NPG) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as per the methods described previously (Scalabrini and others 1998; Otieno and others 2006) with some modifications. Five hundred microliters of 5 mM p NPG prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to 5 mL of each aliquot sample and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 250 μ L cold 0.2 M sodium carbonate (4 °C). The resulting mixture was centrifuged at 14000 \times g for 30 min using an Eppendorf centrifuge (model 5415C; Crown Scientific, Melbourne, Australia) and filtered through a 0.45- μ m membrane filter (Schleicher & Schuell GmbH, Dassel, Germany). The amount of p -nitrophenol released was measured using a spectrophotometer (Pharmacia LKB[®] Novospec II, Uppsala, Sweden) at 420 nm. One unit of enzyme activity is defined as the amount of β -glucosidase activity that released 1 μ mol of p -nitrophenol from the substrate p NPG per milliliter per minute under the assay conditions.

Enumeration of bacterial population

Cell populations of *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 were determined as described previously (Donkor and others 2005). Briefly, 1 g of each fermented soymilk sample was added to 9 mL of sterile 0.15% (w/v) bacteriological peptone (Oxoid) and water diluent and vortexed (model MT 19, Chiltern Scientific, Salmond Smith Biolab Ltd., Auckland, New Zealand) for 30 s. The resulting suspension was serially diluted in sterile 0.15% (w/v) peptone water (Oxoid) and 1 mL of the appropriate dilution was used for selective enumeration by the pour plate technique. The cell growth of each organism was assessed by enumerating bacterial population after 6, 12, 24, 36, and 48 h of fermentation of soymilk on MRS agar (Amyl media). Anaerobic jars and gas generating kits (Anaerobic system BR 38, Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Duplicate plates were incubated anaerobically for 72 h at 37 °C for *L. acidophilus*, *L. casei*, and *Bifidobacterium* spp. Plates containing 25 to 250 colonies were counted and recorded as colony forming units (CFU) per gram of the fermented soymilk.

pH measurements

Changes in pH were monitored during fermentation of soymilk at 0, 6, 12, 24, 36, and 48 h using a pH meter (HANNA Instruments, Singapore).

Production of organic acids in fermenting soymilk

During culture growth, the main metabolic products are organic acids, particularly lactic and acetic acids. The concentrations of these acids were measured according to the method of Donkor and others (2005) using high-performance liquid chromatography (HPLC). The HPLC (Varian Associates, Walnut Creek, Calif., U.S.A.) comprised a solvent delivery system (model 9100) connecting with an auto-sampler (model 9012), a UV light detector (model 9050) and an organic acid analysis column (Aminex HPX-87H, 300 \times 7.8 mm, Bio-Rad Lab, Richmond, Calif., U.S.A.). The mobile phase was 0.001 M H₂SO₄ with a flow rate set at 0.6 mL/min and the temperature of the column was set at 65 °C. Organic acids were detected at 220 nm. For determination of organic acids, 2.5 g samples were mixed with 50 μ L of 15.8 N HNO₃ and 1.0 mL of 0.001 M H₂SO₄ before subjecting a 1.5-mL aliquot of the mixture to centrifugation at 14000 \times g for 30 min at room temperature (~20 °C). The supernatant was filtered through a 0.45- μ m membrane filter (Schleicher & Schuell, Dassel, Germany) and 20 μ L of the filtrate was injected into the HPLC system.

Isoflavone extraction

The extraction of isoflavones from fermented and non-fermented soymilk was performed in triplicate using the method described by Otieno and others (2006) with some modifications. Briefly, 50-mL aliquots of methanol were added to 1.0 g freeze dried sample in a 150-mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was poured into a 50-mL centrifuge tube and was centrifuged at 4000 \times g for 30 min at 10 °C using a Sorvall RT7 refrigerated centrifuge (Newtown, Conn., U.S.A.). A 2-mL aliquot of the supernatant was withdrawn and mixed with 50 μ L of internal standard (ISTD) flavone solution (0.2 mg/mL) and evaporated to dryness using the speed vac concentrator connected to Savant refrigerated trap and vacuum pump (model SC110, Savant Instruments Inc., Farmingdale, N.Y., U.S.A.). The dried sample was dissolved in 1 mL mobile phase (0.05% TFA in 50% of 100 mM ammonium acetate and 50% methanol) and the resulting solution was centrifuged at 14000 \times g for 30 min using an Eppendorf centrifuge (model 5415C; Crown Scientific). The supernatant was

filtered through a 0.45- μ m membrane filter (Schleicher & Schuell GmbH) into an HPLC vial.

Isoflavone standards

Isoflavone standard stock solutions were prepared by dissolving genistein, daidzein, genistin, and flavone (Sigma) in HPLC grade methanol and glycitein, daidzin, and glycitin were dissolved in ethanol (Sigma) to give a 1 mg/mL concentration. Calibration curves were prepared for each standard with 6 different concentrations (0, 20, 40, 60, 80, and 100 μ g/mL). A mixture of the standards was also analyzed and used for identification purposes.

HPLC with electrochemical detection of isoflavone in fermented soymilk

For electrochemical determination of isoflavones in soymilk, a highly sensitive HPLC-ECD method was used as described previously (Klejdus and others 2004) with some modifications. Isoflavone extracted from unfermented (control) and fermented soymilk samples were profiled on a reversed-phase Varian HPLC (Varian Analytical Instruments) system. The HPLC system for electrochemical detection consisted of the following components: a Varian model 9100 auto sampler, a Varian model 9012 solvent delivery system, injector, a Metrohm model 656 amperometric, and 641 VA detector with a 1 μ L cell with glassy carbon working and auxiliary electrode, and a Ag/AgCl reference electrode (Metrohm, Herisau, Switzerland). Samples were applied using a 20 μ L injection loop and separation of isoflavone compounds was achieved using an Alltima HP C₁₈ HL, 5 μ m, 250 x 4.6 mm column and a guard column (Alltech Associates, Inc., Deerfield, Ill., U.S.A.). The isoflavones were eluted by isocratic runs of 40 min with mobile phase (0.05% TFA in 50% of 100 mM ammonium acetate and 50% methanol) at a

flow rate of 1 mL/min and room temperature (\sim 20 °C), a sensitivity range of 0.1 to 50 nA, and an electrochemical detection at +800 mV. All solvents were filtered through a 0.45- μ m membrane filter (Schleicher & Schuell GmbH) and degassed by ultrasound and helium sparging.

Retention times of standards of isoflavones eluted as single peaks (glycitin, daizin, genistin, daizein, glycitein, and genistein) ranged between 2.57 to 17.48 min, and the peak areas were used for quantitation. Calibration runs were prepared for each analytical series with external standards in the range of 20 to 200 ng/mL of isoflavones. The calibration curves were linear with a regression coefficient of 0.998.

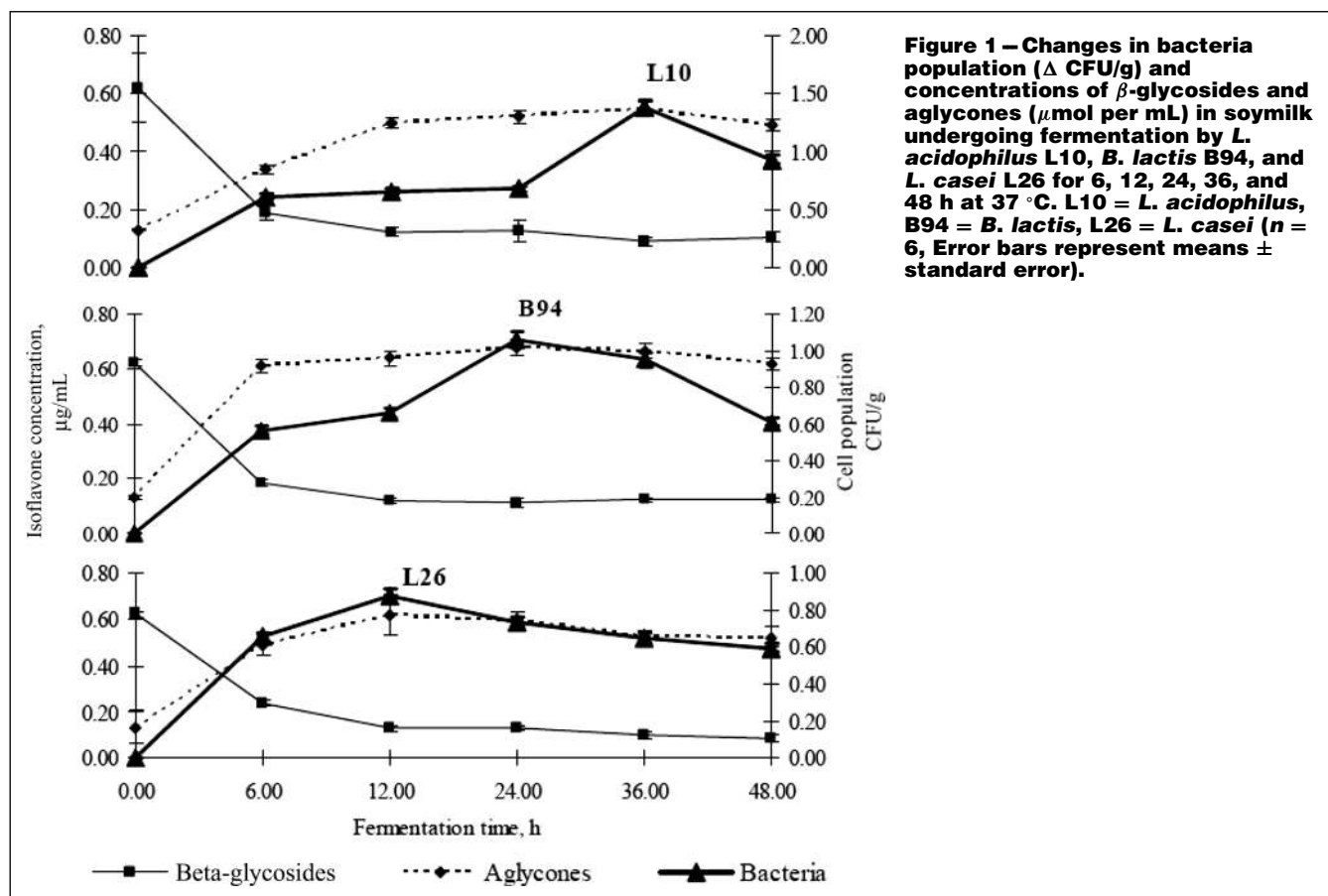
Statistical analysis

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 the Huynh-Feldt (H-F) condition (Littell and others 1998). Where appropriate, 1-way ANOVA and correlational analysis were employed using Microsoft® Excel StatPro™ (Albright and others 1999) and the multicomparison of means was assessed by Tukey's test. The statistical level of significance was preset at 0.05. All experiments were replicated and subsampled at least twice ($n = 6$).

Results

Cell growth during fermentation and storage

Cell populations of *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 in soymilk during fermentation at 6, 12, 24, 36, and 48 h at 37 °C are shown in Figure 1. The highest viable counts



during fermentation occurred at 12 h for *L. casei* L26, 24 h for *B. lactis* B94, and 36 h for *L. acidophilus* L10. Subsequently the populations declined ($P > 0.05$) slowly for each microorganism after growth (Figure 1). Soymilk fermented with *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 and stored at 4 °C for 28 d showed an increased trend of bacterial growth, with *L. acidophilus* L10 and *B. lactis* B94 exhibiting increased population by 20% and 14%, respectively (Figure 2). Even though the different fermentation times influenced bacterial growth during storage at 4 °C, there was a general decline in cell populations during storage from 21 to 28 d.

Decline in pH during fermentation and storage

There was a decline in pH during fermentation at 6, 12, 24, 36, and 48 h at 37 °C (data not shown). Each batch of soymilk showed consistent decrease in pH from the initial pH of 6.58 during 48 h incubation. *B. lactis* B94, and *L. casei* L26 presented a lower but similar drop in pH (0.73 pH units) compared to *L. acidophilus* L10 which showed a decline of 0.59 pH units at the end of storage. The trend was similar and was not significantly different for samples from all

5 fermentation times during the 28 d of storage period (data not shown).

Organic acids concentration during fermentation and storage

The concentration of lactic and acetic acids in fermented soymilk produced by *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 is shown in Figure 3. In general, the lactic acid concentration was higher ($P < 0.05$) than acetic acid for all treatments and times. The concentration of lactic acid in soymilk increased consistently during 48 h of fermentation for all the organisms, with *L. acidophilus* L10 producing the lowest concentration of lactic acid but not significantly ($P < 0.05$) different from *B. lactis* B94. However, in comparison to *L. acidophilus* L10, the production of lactic acid by *L. casei* L26 was significantly ($P < 0.05$) higher at the end of fermentation. Even though the organic acid content increased slightly during storage at 4 °C for 28 d, there was no significant difference between cultures for lactic and acetic acids (data not shown). Since the growth was slow during storage, the production of organic acids was lower ($P > 0.05$) which resulted in a minimal decline in pH.

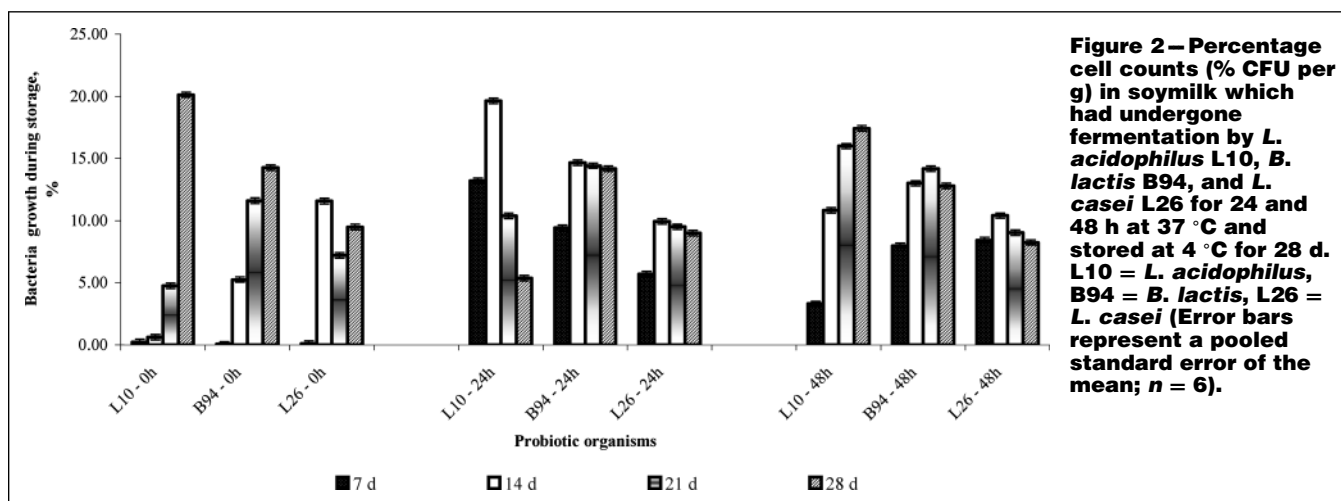


Figure 2—Percentage cell counts (% CFU per g) in soymilk which had undergone fermentation by *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 for 24 and 48 h at 37 °C and stored at 4 °C for 28 d. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei* (Error bars represent a pooled standard error of the mean; n = 6).

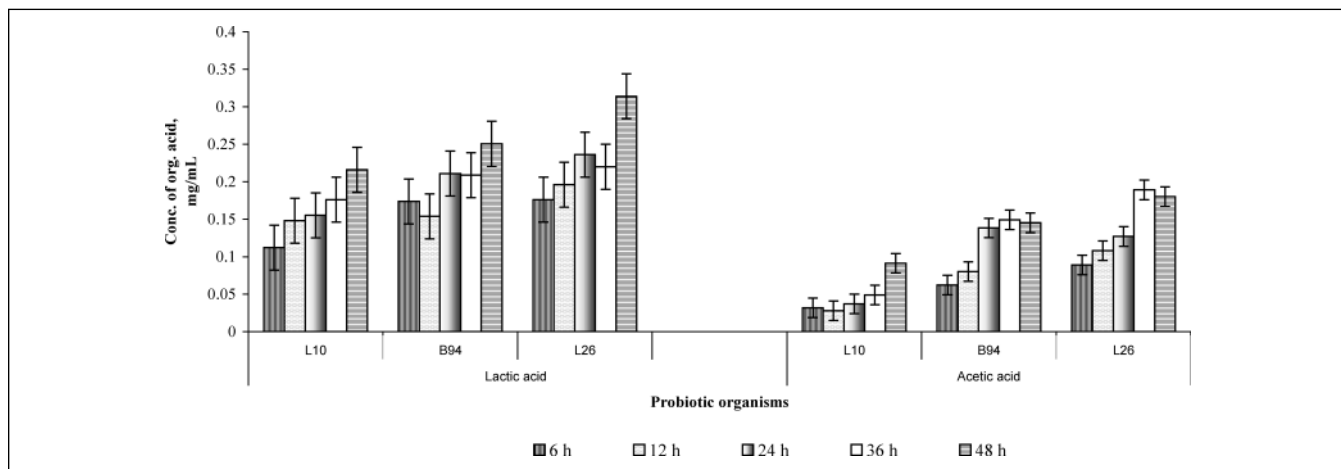


Figure 3—Lactic and acetic acid concentrations produced in soymilk undergoing fermentation by *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 for 6, 12, 24, 36, and 48 h at 37 °C as single cultures. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei* (error bars represent a pooled standard error of the mean; n = 6; SEM for lactic and acetic acid = 0.03 and 0.01 mg/mL, respectively).

Consequently fermented soymilk did not set during 28 d storage at 4 °C.

β -glucosidase activity

The β -glucosidase activity of *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 in soymilk before and after fermentation at 37 °C is shown in Figure 4. The maximum yield of β -glucosidase activity varied ($P < 0.05$) between all 3 organisms. *L. acidophilus* L10 showed increased enzyme activity at 36 h of fermentation whereas *B. lactis* B94 and *L. casei* L26 attained maximum enzyme activities at 24 and 12 h, respectively. During storage at 4 °C, all microorganisms showed maximum β -glucosidase activity ranging from 0.117 to 0.204 $\mu\text{mol}/\text{mL}$ at d 21 irrespective of fermentation time. Overall, *B. lactis* B94 exhibited the highest β -glucosidase activity followed by *L. acidophilus* L10 during storage (data not shown). The correlation between β -glucosidase activity and growth of microorganisms ranged from 0.70 for *L. acidophilus* L10 to 0.79 for *B. lactis* B94, which further suggested the effects of growth of microorganisms in soymilk on β -glucosidase activity.

Hydrolysis of isoflavone compounds in fermented soymilk by microorganisms

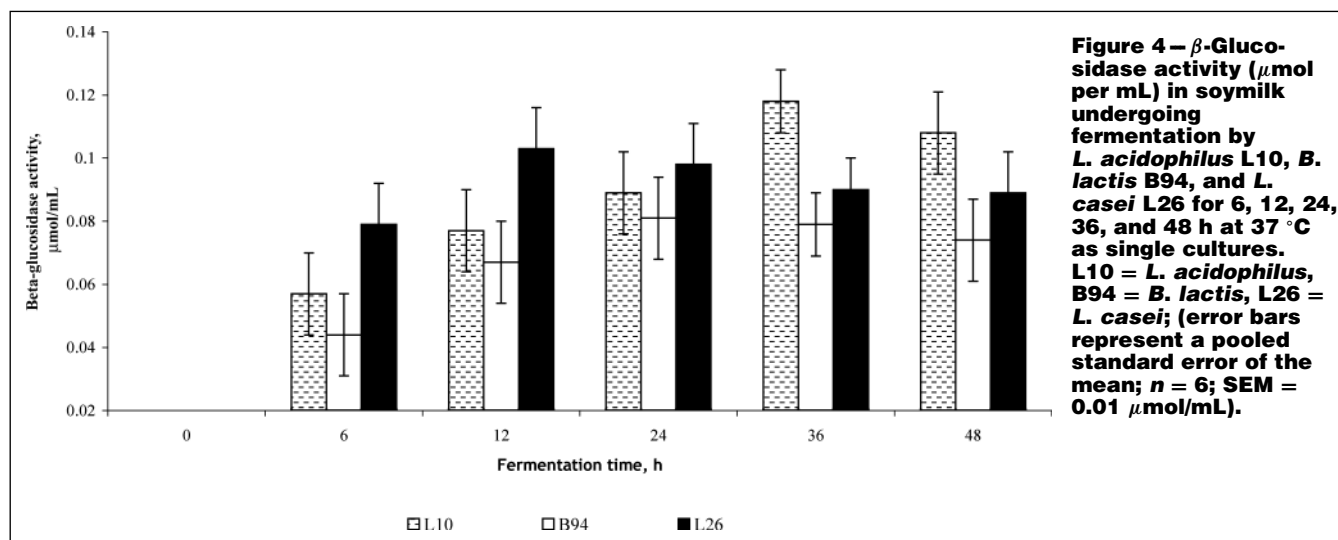
The changes in isoflavone concentration in relation to increasing bacteria population in soymilk during 48 h fermentation at 37 °C with *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26, respectively, is shown in Figure 1. The maximum concentration of aglycones produced corresponded to the maximum cell population of each microorganism. In general, the concentration of isoflavone aglycones increased ($P < 0.05$) and at the same time the concentration of β -glycosides was reduced ($P < 0.05$) compared to that in unfermented soymilk. However, the concentrations of malonyl- and acetyl-glycosides were relatively low and there was no significant change ($P > 0.005$) during the 48 h fermentation (data not shown). There was an appreciable increase in the concentration of daidzein, glycitein, and genistein from 6 h of incubation and they continued to increase up to 36 h for *L. acidophilus* L10, 24 h for *B. lactis* B94, and 12 h for *L. casei* L26. *L. acidophilus* L10 yielded a 63% increase in aglycone concentration at 36 h, whereas *B. lactis* B94 and *L. casei* L26 showed 77% and 71% increase in aglycones at 24 and 12 h, of incubation, respectively, compared to nonfermented soymilk (Figure 1).

Discussion

All cultures showed good growth characteristics in soymilk, indicating that soymilk was a good delivery medium for probiotic organisms. Previous studies have shown that these bacteria produce α -galactosidase required to metabolize raffinose and stachyose and grow well in soymilk (Scalabrini and others 1998; Donkor and others 2006). The growth of the organisms declined after the respective peak growth period, possibly due to diminishing nutrient supply in the medium. A similar trend of cell population decline was observed during storage at 4 °C for 28 d.

During soymilk fermentation, the main metabolic end products include organic acids. These lead to a reduction in the pH of soymilk. Previous studies have reported similar findings (Angeles and Marth 1971; Kamaly 1997; Liu 1997). Acetic acid is an undesirable end product in fermented soymilk due to its "vinegary" flavor. Therefore the high production of lactic acid over acetic acid by lactobacilli and bifidobacteria in this study was desirable for the production of fermented soymilk. Similar results were reported by Donkor and others (2005) in soy yogurt made with commercial soymilk and fermented by *L. acidophilus*, *Bifidobacterium*, and *L. casei* and during storage at 4 °C.

The respective maximum β -glucosidase activities observed for each culture showed that the period of fermentation at which the enzyme activity was highest corresponded to their cell populations. The increased β -glucosidase activity between 24 and 36 h was significant ($P < 0.05$) for *L. acidophilus* L10, which had the highest β -glucosidase activity compared to *B. lactis* B94 and *L. casei* L26. Otieno and others (2006) also reported that *L. acidophilus* showed the highest β -glucosidase activity at 24 h of fermentation in soymilk compared to *Bifidobacterium* spp. and *L. casei*. Generally, β -glucosidase activity was found to be strain ($P < 0.0436$) and time ($P < 0.0001$) dependent and, therefore, the enzyme activity varied among the organisms used during the 48 h fermentation at 37 °C and this pattern continued throughout storage at 4 °C. *L. acidophilus*, *Bifidobacterium*, and *L. casei* were reported to show strain-dependent β -glucosidase activity in soymilk (Tochikura and others 1986; Otieno and others 2005). The β -glucosidase enzymes produced by these microorganisms are responsible for the breakdown of β -1-6 glucosidic bond, which conjugates the pran ring of isoflavone and the sugar moieties. These enzyme activities significantly increased the content of isoflavone aglycones in fermented soymilk after incubation with *L. acidophilus*, *Bifidobacterium*, and



L. casei as compared to that in unfermented soymilk. The breakdown of isoflavone glycosides into sugar moieties and bioactive isoflavone aglycones during fermentation could improve the biological activity of soymilk.

Conclusions

L. acidophilus L10, *B. lactis* B94, and *L. casei* L26 showed varying levels of β -glucosidase activity, which depended on the growth of each organism. In addition, β -glucosidase activity was strain dependent. *L. acidophilus* L10, *B. lactis* B94, and *L. casei* L26 showed 20%, 15%, and 11% greater cell counts compared to the initial numbers, respectively. Increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of isoflavone aglycones in fermented soymilk, compared to unfermented soymilk. Increased isoflavone aglycone content in fermented soymilk is likely to improve the biological functionality of soymilk.

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