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PERFORMANCES OF NEW ISOLATES OF *BIFIDOBACTERIUM* ON FERMENTATION OF SOYMILK

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Growth and metabolic activity of several new, human origin isolates of *Bifidobacterium* strains were investigated. All tested bifidobacteria strains were grown well on the native soymilk medium without any additional nutrients. The fermentation processes cultured with initial cell concentrations in 10^5 – 10^7 cfu/ml resulted in 10^8 cfu/ml after 8–12 h of incubation in soymilk, and were kept viable up to the end of fermentation (48 h). Volumetric productivities of *B. bifidum* B3.2, *B. bifidum* B7.1 and *B. breve* B9.14 were 1.6×10^{10} cfu/L.h, 4.5×10^{10} cfu/L.h and 7.6×10^9 cfu/L.h, respectively, whereas these values of *B. lactis* Bb-12 and *B. longum* Bb-46 probiotic strains were 2.7×10^9 cfu/L.h and 1.0×10^{10} cfu/L.h. The α -galactosidase activities were also detected in the intracellular fraction of the disrupted cells. Productions of lactic and acetic acids were in the range of 23–60 mmol/L and 2.4–5.6 mmol/L, respectively. Molar ratios of acetate to lactate in all tested strains varied from 0.05–0.1 that are very promising for further technological development of probiotic fermented soy-based food products.

Keywords: bifidobacteria, soymilk, short chain fatty acid (SCFA), fermentation, probiotic, α -galactosidase, galacto-oligosaccharides

Introduction

Soybean (*Glycine max. L. Merr*) is one of the most important oleaginous seeds in the world due to availability and rich in high quality proteins, essential amino acids, calcium, phosphorus, iron, vitamins (especially A and B) and vegetable oil [1]. Soy-based foods may provide a range of health benefits through

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hypolipidemic, anticholesterolemic and antiatherogenic properties as well as reduction in allergenicity [2, 3] and risk of most hormone-associated health disorders [4]. Evidences are available that consumption of soy-derived food had potential health benefits related to cardiovascular diseases, menopausal symptoms, osteoporosis, breast and prostate cancers because they are rich sources of bioactive phenolic compounds [5, 6]. Moreover, soybean contains all amino acids essential to human nutrition, thus it should be a good food base for substitution of milk for those who are vegetarians or lactose-intolerant [7, 8]. However, consumption of soymilk is hindered due to the presence of unpleasant off-flavours carried over from soybean as well as various oligosaccharides including raffinose and stachyose that may cause a gastrointestinal discomfort known as flatulence to consumers [9]. Raffinose and stachyose are non-digestible α -galactosidic oligosaccharides due to lack of α -galactosidase in the human gastrointestinal tract. These matters can be addressed and eliminated by treatment using external α -galactosidase enzyme or/and fermentation with microorganism possessing high α -galactosidase activity. The latter concept should be more attractive from all scientific, technical and nutritional points of view, because in one processing step there is possibility to remove off-flavour effects causing by *n*-hexanal and pentanal (mainly occurring in beans and formed from unsaturated fatty acids) [9]; and production of probiotic products with high nutritional values. In the last few decades, intensive research dealing with fermentation of soymilk using mainly *Lactobacillus* and *Bifidobacterium* strains was carried out worldwide [10–14]. Hydrolysis of isoflavones as well as production of flavourful lactic acid during fermentation of soymilk was reported by Bordignon et al. in 2004 [15]. Reduction of galacto-oligosaccharides (raffinose, stachyose, soygalacto-oligosaccharides, etc.) by various lactic acid bacteria (*Lb. cellobiosis*, *Lb. plantarum*, *Lb. curvatus*, *Lb. fermentum*, *Lb. pentosus*, etc.) have also been reported by several authors [16].

Nowadays, several *Bifidobacterium* strains are well known as probiotics with many health-promoting effects, and they play an important role in the microbial ecology of the human and animal gut [17–24]. Moreover, some strains of this genus were also reported to be capable of metabolising α -galactosyl type galacto-oligosaccharides [25, 26], thus soymilk that contains sucrose, raffinose, stachyose, proteins, vitamins, etc., should be a good medium for growing bifidobacteria [25]. This hypothesis was also proved by Bordignon et al. [15] when they reported that *B. bifidum* JCM 1255, *B. breve* JCM 1922 and *B. infantis* JCM 1222 strains preferentially fermented galacto-oligosaccharides rather than sucrose, during fermentation of soymilk. Although, due to α -galactosidase activity, *Bifidobacteria* are able to cleave α -galactosidic bonds, very few data are available in the literature related to properties of the α -galactosidase enzyme from this

micro-organism. Generally, *Bifidobacterium spp.* utilise glucose through the so-called “bifidus pathway” resulting in high levels of short chain fatty acids (SFCA) including lactic, propionic, butyric, acetic acid, etc. Meanwhile, lactic acid is a flavourful compound, whereas the presence of acetic acid causes odour defects in the final product, and thus it could be a main drawback of application of bifidobacteria in a fermentation system. However there is no doubt that the molar ratio of lactic and acetic acid varies from species to species, even from strains to strains and also fermentation conditions. In the case of lactic acid bacteria, some authors [27, 28] reported that an increased supplement of nitrogen source resulted in higher concentrations of lactic acid, and thus the flavour of a product should be better. In our previous study [29], this effect was also observed when studying fermentation of carrot juice with several *Bifidobacterium* strains. In this study, performances of newly isolated, human origin *Bifidobacterium* strains on the fermentation of soymilk were focused.

Materials and Methods

Media

Trypticase–Phytone–Yeast medium (TPY) contained (per litre) trypticase (BBL) 10 g, phytone (BBL) 5 g, glucose 5 g, yeast extract (Difco) 2.5 g, Tween 80 1 ml, L-cysteine HCl 0.5 g, K_2HPO_4 2 g, $MgCl_2 \cdot 6H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2$ 0.15 g, $FeCl_3$ 0.03 g, pH ca. 6.0.

Soymilk: Soybeans were washed and soaked in water for one day at room temperature. Soaking water was drained and beans boiled in fresh water (its quantity was the quadruple of the soybeans) for 30 minutes, then the whole amount was crushed with mixer for 5 minutes and filtered through double-layer cloth to yield soymilk. The cake was extracted several times to gain approximately 4.5–5 litres soymilk per 500 g soybean. It was autoclaved at 121 °C for 15 minutes.

Microorganisms and their cultivation

Bifidobacterium lactis Bb-12 and *B. longum* Bb-46 were purchased from Chr. Hansen A/S (Hørsholm, Denmark). *Bifidobacterium bifidum* B3.2, *B. bifidum* B7.1 and *B. breve* B9.14 were isolated from human faeces and identified [30]. All *Bifidobacterium* strains were pre-cultured anaerobically (in Bugbox anaerobic chamber, Ruskin Technology) in TPY medium at 37 °C for approximately 24 h.

Fermentation

Fermentation was initiated with 10^6 – 10^7 CFU (colony forming unit)/mL concentration of the relevant *Bifidobacterium* strains. All trials were carried out under anaerobe conditions in Anaerobe Jar+GasPak System (OXOID) or in Bug-box anaerobic chamber at 37 °C. Fermentation was followed by determining viable cell counts, measuring pH and titratable acidity.

Analytical procedures

Titratable acidity (SH°) and pH: The titratable acidity was determined with Soxhlet-Henkel method by titration. During cultivation, the main metabolic products are organic acids, particularly lactic and acetic acids. The pH changes in batches of soymilk or TPY media were monitored during fermentation using a SevenMulti pH-meter (Metler Toledo, USA).

Viable counts: The plate counts of bifidobacteria were determined on Beeren's agar [29] or TPY agar. Samples from the fermented broth were diluted by 10-fold serial dilution and aliquots were transferred into Petri dishes and mixed with the molten cooled medium (37 °C). After solidification, the plates were incubated under anaerobic conditions at 37 °C. The colonies were counted after 48 h or 72 h incubation.

Determination of organic acids: The concentrations of organic acids were determined with Waters HPLC System consisting of W610 pump Waters HPLC Controller, 717 plus autosampler W410 refractive index (RI), and photodiode array (PDA) detectors. A thermostatically controlled column compartment set at 45 °C containing Aminex HPX-87H ion exclusion column was used at a flow rate of 0.6 mL/min using 5 mM H₂SO₄ as the mobile phase. The data acquisition and integration were performed using the Millennium™4.0 software package. Each sample was injected three times. Standards (external and internal) of organic acids (lactic, acetic, malic, citric, succinic, H₂SO₄ and oxalic) were used to identify and quantify the components in the samples.

Enzyme activity assay: The α -galactosidase enzyme activity was assayed in the reaction mixture containing 0.3 mL of McIlvaine buffer (100mM, at pH 6.6) and 0.5 mL of 15 mM *p*-nitrophenyl α -D-galactopyranoside (pNP α Gal) substrate. The cells were disrupted by a chemical method (Cetyl trimethylammonium bromide, CTAB) and the intracellular fraction was used for assaying enzyme activity. Buffered substrate was pre-incubated at the relevant temperature for 5 min and the enzyme reaction was started by adding 0.2 mL of adequately diluted enzyme solution. After 5 min the enzyme reaction was stopped by add-

ing 5 mL of 0.1M Na₂CO₃ solution. The released p-nitrophenol was determined spectrophotometrically at 405 nm using linear calibration prepared with p-nitrophenol under the same conditions.

One unit (U) of enzyme activity was defined as the amount of enzyme that releases one μmol p-nitrophenol per min under the standard conditions.

Statistical analysis

All data are presented as the mean and standard deviation (SD). One-way analysis of variance (ANOVA), unpaired and paired Student's *t*-tests were done using Statistica v9.0 software package (StatSoft, USA). Generally, only $p < 0.05$ was accepted as the statistical significance level.

Results and Discussion

Growth, volumetric productivity and α-galactosidase activity of bifidobacteria

The most important factor in developing technology for the production of probiotic foodstuffs is growth and viability of applied strain(s). The international standards recommend that the fermented products claiming health benefits must contain a minimum of 10⁷ viable probiotic bacteria per gram of product at the time of purchase [29]. The changes in viable cells of bifidobacteria during fermentation of soymilk are presented in Figure 1. Generally, maximum counts of cell numbers occurred at the 12th hour of fermentation except for *B. bifidum* B3.2 strain in independent of the initial cell concentration (from about 5×10⁶ cfu/mL to 10⁷ cfu/mL). The counts of most strains varied from 5×10⁷ to 10⁸ cfu/mL, in the case of *B. bifidum* B7.1 about 5×10⁸ cfu/mL were counted as in the case of *B. bifidum* B3.2 at 24 h of fermentation of soymilk. These numbers are significantly higher than the counts of *B. lactis* Bb-12 and *B. logum* Bb-46 that are probiotic strains. Both *B. bifidum* B3.2 and B7.1 strains are the new isolates and deposited in the National Collection of Agricultural and Industrial Microorganisms, Budapest. Shimakama et al. [36] also reported that *B. breve* strain Yakult needed about 12 hours to reach stationary phase with about 4×10⁹ cfu/mL (from about 5×10⁷ cfu/mL). Our results differ from those reported by Hou et al. [37] or Wang et al. [13] when they studied growth of *B. infantis* and *B. longum* in soymilk.

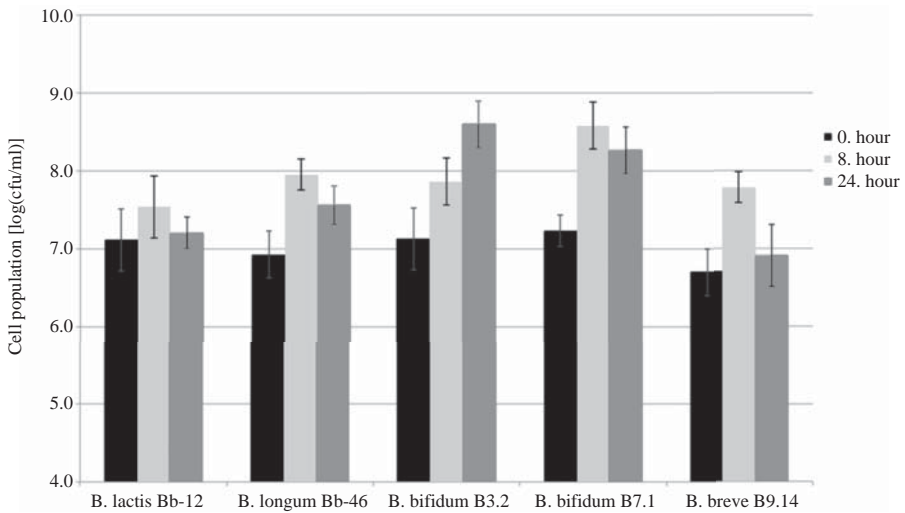


Figure 1. Growth of *Bifidobacterium* spp. in soymilk

They found that both *B. infantis* CCRC 14633 and *B. longum* B6 strains take at least 24 hours to reach maximum cell numbers in native soymilk.

Volumetric productivities of *B. bifidum* B3.2, *B. bifidum* 7.1 and *B. breve* B9.14 were 1.6×10^{10} cfu/L.h, 4.5×10^{10} cfu/L.h and 7.6×10^9 cfu/L.h, respectively. These values were significantly higher than those of probiotic strains *B. lactis* Bb-12 (2.7×10^9 cfu/L.h) and *B. longum* Bb-46 (1.0×10^{10} cfu/L.h) as well as were not far from volumetric productivities of some bifidobacteria in carrot juice [29]. In the case of *B. lactis* Bb-12 strain, productivity was less in soymilk (2.7×10^9 cfu/L.h) compared with MRS medium (1.1×10^{10} cfu/L.h) [38]. Generally, the volumetric productivity of bifidobacteria in soymilk is less than in milk, even when the same strains are used [11, 39, 40]. Our results are also in agreement with this observation.

Minimal decreases in saccharides concentration during fermentation were detected (data are not shown). Interestingly, few data are available in the literature about α -galactosidase from *B. lactis* Bb-12 and *B. longum* Bb-46 that are widely applied as probiotics strains in the dairy industry. In fact, in milk based products, lactose is present as the main sugar, and bacteria should synthesise β -galactosidase to utilise it. In soymilk, galactose based-(oligo)saccharides (mainly α -galactosides) are an important part of the total carbohydrate content, thus to grow on this type substrate, the bifidobacteria have to secrete α -galactosidase enzyme. Desjardins et al. [31] observed that bifidobacteria possessed high activi-

ties of α -galactosidase and β -galactosidase using API ZYM kit. *Bifidobacterium adolescentis* DSM 20083 strain secreted α -galactosidase extracellularly; that was suggested to be a tetrameric structure of the protein [32]. Later, two groups – Goulas et al. [33] as well as Zhao et al. [34] – reported cloning, expression and characterisation of α -galactosidase from *B. bifidum* NCBIM B41171 and *B. breve* 203, respectively, and it had been found a monomeric nature of this galactosidase with molecular mass about 80 kDa. Tochikura et al. [35] however, found that bifidobacteria exhibit higher hydrolyzing activity toward various p-nitrophenyl glycosides than other intestinal bacteria. In our study, the raffinose as the inducer was applied to check the production of α -galactosidase by new isolates of the bifidobacteria. The laboratory media were supplemented with 1% (mass per volume) raffinose and then cultured with ca. 10^7 cfu/ml of different *Bifidobacterium* strains. Not any α -galactosidase activities were measured in the ferment broth (the extracellular fraction). After disruption of bacterial cells, the α -galactosidase activity was detected meaning the bacteria synthesized it intracellularly. Similar α -galactosidase activity was observed the *B. bifidum* B7.1 strain comparing with the *B. lactis* Bb-12 (3×10^{-11} U/cfu). In the cases of the *B. bifidum* B3.2 and the *B. breve* B9.14 strains low α -galactosidase activities were observed, even they were grown very well.

Changes in titratable acidity and pH

During fermentation of soymilk, the titratable acidity (TA) increased from about 3 SH° to the range 14.27–29.32 SH° depending on the strain used; meanwhile the pH value decreased from 6 to 4.5 (Figure 2). The highest acidity (29 SH°) was observed in the case of *B. bifidum* B3.2 strain. Intensive growth of bacteria was also confirmed by the drop in pH in the first 8 hours of fermentation (Figure 2). In general, pH values dropped from 6.0 to 5.0 or below. In 2003, Wang et al. [13] reported that in similar situations, *B. infantis* CCRC 14633 takes about 40 hours and 48 hours of fermentation to reach pH 5.04 and pH 4.61, respectively. *Bifidobacterium breve* JCM 1192 strain was reported to achieve a better fermentation profile (drop in pH from 6.2 to 5.1 after 16 h), while *B. adolescentis* JCM 1275 and *B. bifidum* JCM 1255 produced less acid during fermentation of soymilk [15]. Our results are comparable to some profiles produced by lactic acid bacteria such as *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO 13953 [15]. *Lb. casei* subsp. *rhamnosus* FNCC 098, *Lb. plantarum* SMN 25, *Lb. plantarum pentosus* SMN 01 [41].

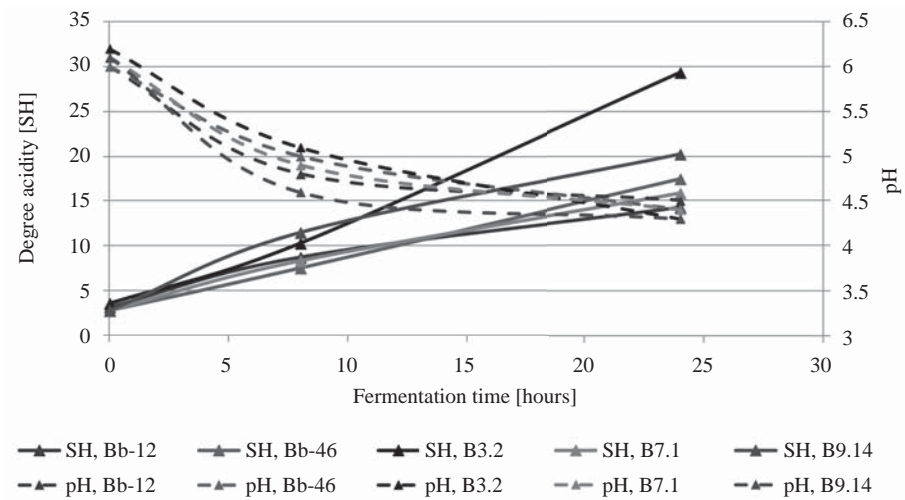


Figure 2. The profile of the acidity and pH of the soymilk fermentation with bifidobacteria

Changes in lactic, acetic and propionic acid concentration

It is well known that lactic acid is one of most important compounds in formation of the flavor of fermented products such as soymilk. One unique aspect of bifidobacteria is that all the lactic acid produced is in the L(+) form which is more easily metabolized by infants than the D(-) form [42]. Theoretically, utilisation of carbohydrates through the “bifidus” pathway, the bifidobacteria, produces more acetic acid than lactic acid (generally 3:2 in molar ratio) [43]. Changes in some organic acids in soymilk during fermentation with four applied *Bifidobacterium* strains are summarized in Table I. Due to intensive metabolic activity, both new isolates: *B. bifidum* B7.1 and *B. bifidum* B3.2 strains produced high amount of lactic and acetic acids in fermentation of soymilk even after 6 hours (in the case of B7.1 51.36 mmol/L lactic and 2.65 mmol/L acetic acid, in the case of B3.2, 43.61 mmol/L and 3.43 mmol/L, respectively). The lactic acids content in the fermented soymilk increased to 58.14 mmol/L and 58.55 mmol/L in the cases of *B. bifidum* B7.1 and B3.2 strains, respectively, whereas about the increase of acetic acid was about 2 mmol/L after 12 of fermentation process. The production of short chain fatty acids by the new isolates are comparable with commercial strain *B. lactis* Bb-12 and significantly better than *B. longum* Bb-46. Additionally, our results were about ten times higher than those reported by Donkor et al. [44]. In their study, *B. lactis* B94 and *B. longum* B1536 produced 0.02 and 0.03 mg/mL lactic acid and 0.05 mg/ml acetic acid concentration at the 12th hours of soymilk fermentation. Higher acetic acid concentrations were

Table I. Changes of lactic, acetic and propionic acid in soymilk fermented with different *Bifidobacterium* strains

Fermentation time (h)	Content (mmol/L)			Molar ratio (acetic/lactic)
	Lactic acid	Acetic acid	Propionic acid	
<i>B. bifidum</i> B3.2				
0	4.22±0.73	0.72±0.52	0.22±0.21	0.17
6	43.61±0.66	3.43±0.81	4.27±0.25	0.08
12	58.14±0.48	5.57±0.71	5.23±0.25	0.10
<i>B. bifidum</i> B7.1				
0	4.22±0.67	0.72±0.47	0.22±0.35	0.17
6	51.36±0.69	2.65±0.25	3.58±0.41	0.05
12	59.55±0.99	4.59±0.62	3.98±0.29	0.08
<i>B. lactis</i> Bb-12				
0	4.22±0.51	0.72±0.44	0.22±0.32	0.17
6	52.52±0.78	4.44±0.39	1.03±0.37	0.08
12	56.35±0.25	5.01±0.42	2.75±0.81	0.09
<i>B. longum</i> Bb-46				
0	4.22±0.55	0.72±0.33	0.22±0.27	0.17
6	16.12±0.61	1.62±0.71	0.75±0.24	0.10
12	23.68±0.84	2.43±0.66	0.82±0.24	0.10

published by Hou et al. [37], where they found that 11.32 mmol/L and 11.42 mmol/L acetic acid concentrations were determined after 12 h of fermentation using *B. infantis* CCRC 14633 and *B. longum* B6 strains, respectively. Based on molar ratio of acetic to lactic acid calculated, in the first stage of fermentation these values were about 1.8–1.9, thus *B. infantis* CCRC 14633 and *B. longum* B6 did not follow only the bifidus pathway. Interestingly, all of investigated strains in our study produced much more lactate than acetate in molarity (0.05–0.10 molar ratio). Our present results confirm data reported in our previous study [29] on fermentations of carrot juice. Some studies dealing with nutrients necessary for lactic acid fermentation have been established [27–28]. They found that supplementing with more nitrogenous components resulted in higher concentrations of lactic acid. Soymilk is rich in protein and amino acid content, thus it may result in changes of molar ratio of acetate to lactate during fermentation. Furthermore, these results demonstrated that the molar ratio of acetic and lactic acids

produced by bifidobacteria varies depending on numerous parameters such as applied strains, culture medium, the fermentation time, even fermentation conditions, etc.

Conclusions

All investigated human origin *Bifidobacterium* strains (*B. bifidum* B3.2, *B. bifidum* B7.1 and *B. breve* B9.14) were able to grow and ferment native soymilk without any nutrient supplementation like the commercial probiotic ones *B. lactis* Bb-12 and *B. longum* Bb-46. These strains produce high levels of lactic acid in fermented soymilk that resulted in titratable acidities in the range of 14 to 29 SH°. Moreover, the molar ratios of acetate to lactate concentration at 12 h of fermentation varied from 0.05 to 0.1 that are very good results from technological points of view, since high concentration of acetic acid causes odour defects in the final product. Overall, the performances of newly isolated, human origin *Bifidobacterium* strains on fermentation of native soymilk were comparable with the industrial ones, thus our results are very promising and may serve as base for developing the technology for production of probiotic fermented soymilk.

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Conflict of Interest

No conflict of interest.

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