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Fermentation of Soymilk by Yoghurt and Bifidobacteria Strains

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Abstract

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Soy and milk based products fermented by yoghurt culture YC-381 alone or in combination with two probiotic cultures (*Bifidobacterium animalis* subsp. *lactis* BB 12 and *Bifidobacterium bifidum* CCDM 94) were prepared. Bacteria growth, the amounts of lactic acid, acetic acid, and acetaldehyde were compared after 16 h of fermentation at 37°C. The changes of isoflavones concentrations were also monitored in soy products. The growth of *Streptococcus thermophilus* and bifidobacteria was similar in both media, but *Lactobacillus delbrueckii* subsp. *bulgaricus* showed a better growth in milk. Titratable acidity and the concentrations of acids were consistently higher in cow milk than in soymilk at the end of fermentation. Bifidobacteria, compared to the yoghurt culture, were only able to acidify the media to the half values. Comparing the bifidobacteria strains, *Bifidobacterium animalis* subsp. *lactis* BB 12 exhibited a better ability to acidify milk. The strain *Bifidobacterium bifidum* CCDM 94 was able to release 6.90 mg/100 ml of isoflavone aglycones in soymilk.

Keywords: acetaldehyde; acetic acid; isoflavones; lactic acid

The fermented soy products represent an interesting alternative to the fermented milk products. The metabolism of lactic acid bacteria (LAB) shows some differences between soymilk and cow milk as a result of their different media compositions. The higher concentration of proteins in cow milk increases the buffering capacity of media which is beneficial for the growth of bacteria. Soymilk – lactose free medium – contains galactooligosaccharides which are considered as prebiotics, i.e. compounds supporting probiotic bacteria growth (Farnworth 2007). The metabolism of saccharides and proteins caused by LAB can influence both the nutritional value and final sensory quality of the fermented products. On the other hand, Europeans are not familiar with the organoleptic quality of fermented soy products (Ang *et al.* 2003).

The yoghurt culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) exhibits symbiotic character of growth in cow milk but the support of the growth was not observed in soymilk (Chumchuere & Robinson 1999). *Bifidobacterium* spp. normally inhabits the human gastrointestinal tract and is one of the main probiotic cultures (Shah 2007). As an example of the positive health effects of bifidobacteria can be their ability to bio-activate some substances which can increase their impact on human body.

Isoflavones belong to a group phytoestrogens with the highest level of estrogenic activity. In plants, they occur as free aglycones or are very commonly bonded with glycosides or their malonyl- and acetyl-conjugates. Isoflavone glycosides derived from soymilk require bacterial-induced hydrolysis in order to be transformed into a bioavailable aglycone form (Tsangalis *et al.* 2003). Izumi *et al.* (2000) and Setchell *et al.* (2001) discovered that isoflavone aglycones were absorbed faster and in higher amounts than their respective β-glucoside forms.

The reduction of the beany flavour of soymilk, which improves the sensory quality of the final product,

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could be also connected with fermentation. Bifidobacteria strains, specifically, are able to metabolise *n*-hexanal and pentanal in soymilk – the former is responsible for the undesirable flavour (Desai *et al.* 2002). Furthermore, fermentation also reduces the content of soy galactooligosaccharides: indigestible carbohydrates, which can in higher amounts cause undesirable flatulence (Scalabrini *et al.* 1998).

The aims of the present study were to compare the growths of the yoghurt culture and two different strains of bifidobacteria in cow milk and soymilk, to evaluate the production of short fatty acids and acetaldehyde, and to confirm the biotransformation of isoflavone glycosides to aglycons during the fermentation process.

MATERIAL AND METHODS

Microorganisms. *Bifidobacterium animalis* subsp. *lactis* BB 12 – commercial probiotic strain (Ch. Hansen, Hørsholm, Denmark); *Bifidobacterium bifidum* CCDM 94 (Culture Collection of Dairy Microorganisms; Laktoflora®, Prague, Czech Republic); Yoghurt culture YC-381 – commercial culture (Ch. Hansen, Hørsholm, Denmark).

Preparation of model fermented products. As the cultivation media, whole-fat UHT cow milk (Madeta, Planá nad Lužnicí, Czech Republic) and soymilk Sunfood klasik (Sunfood, Dobruška, Czech Republic) were used after sterilisation at 100°C for 20 minutes. The appropriate amounts (0.5 g) of lyophilised cultures (*B. animalis* subsp. *lactis* BB 12 and yoghurt culture YC-381) were dissolved in 100 ml of soymilk or cow milk which were then stored at 4°C for 12 h to activate the cultures with the aim to shorten their lag phase. The activated microorganisms or the liquid culture (*B. bifidum* CCDM 94) were transferred in the amount of 1% (v/v) to sterile soymilk or cow milk. The inoculated media were cultivated at 37°C for 16 h with the aim to enhance the growth of bifidobacteria. The number of cells at the beginning of fermentation was as follows: *S. thermophilus* 10^6 CFU/ml, *L. delbrueckii* subsp. *bulgaricus* 10^4 CFU/ml, and *Bifidobacterium* spp. 10^6 CFU/ml. All experiments were replicated twice and all results are the mean from three independent measurements (n = 6).

Determination of cell count. The number of *L. delbrueckii* subsp. *bulgaricus* in the samples was determined after 72 h of incubation at 37°C in 4% (v/v) CO_2 atmosphere on MRS agar (Oxoid, Hampshire, UK), pH 5.2; the number of *S. thermophilus* after 48 h of incubation at 37°C on M17 agar (Oxoid, Hampshire, UK), pH 6.8, and the number of *Bifidobacterium* spp. after 4 days at 37°C in an anaerobic jar with an anaerobic gas generating kit AnaeroGen on MRS agar (both Oxoid, Hampshire, UK) with 0.5 g/l L-cysteine (Merck, Darmstadt, Germany) and 2 mg/l dicloxacilin (Sigma, St. Louis, USA), pH 6.8.

Physico-chemical analyses. Total solids were determined by Halogen Moisture Analyzer HR 73 (Mettler Toledo, Greifensee, Switzerland). The fat content was measured according to EN ISO 1211:2010 (Milk – Determination of fat content – Gravimetric method (Reference method). The total nitrogen content was evaluated using a Kjeltec analyzer (Kjeltec 8400 Analyzer; FOSS Analytical AB, Höganäs, Sweden); the protein content was calculated as total nitrogen multiplied by 6.38 for cow milk and 5.80 for soymilk. Ash content was determined by samples incineration in the furnace Ht40P (Lac, Rajhrad, Czech Republic) at 525°C.

pH and titratable acidity. The pH values were determined with a pH meter (Jenway, Staffordshire, UK) provided with a combined electrode. The titratable acidity was evaluated by titration of 25 g of product with 0.1 mol/l NaOH solution to pH 8.3 with phenolphthalein indicator and expressed as mmol H^+/kg. All experiments were replicated twice.

Measurement of metabolic products. Enzymatic kits (Megazyme, Bray, Co., Wicklow, Ireland) were used for the determination of the content of lactic acid isomers (D,L-lactic acid assay), acetic acid and acetaldehyde according to the manufacturer's instruction. All experiments were replicated twice.

Extraction of isoflavones from soymilk. The soymilk or the fermented soy product (1 ml) with chrysin 10 ng/ml (Sigma-Aldrich, Seelze, Germany) as an inner standard was extracted two times with 6 ml of ethylacetate. The solution was centrifuged at 11 000 rcf (Rotina 35 R; Hettich Zentrifugen, Tuttlingen, Germany) for 5 min and the supernatant was dried in a rotary evaporator (BUCHI Rotavapor R-114; Büchi, Flawil, Switzerland) under nitrogen. The dried pellet was resuspended in 10 ml of 50% (v/v) methanol and then diluted 10 times with 50% (v/v) methanol. The extract was filtered through microfilter and transferred to vials for UPLC-MS/MS analysis.

HPLC analysis of isoflavones. The separation was performed using an Acquity Ultra-Performance LC system (UPLC) (Waters, Milford, USA) with an

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Acquity UPLC BEH C18 column (50 mm × 2.1 mm *i.d.*, 1.8 µm particle size; Waters, Milford, USA) connected to a 5500 QTRAP tandem mass spectrometer (AB SCIEX, Toronto, Canada), equipped with an TurboIon™ electrospray (ESI) ion source operated in both positive and negative modes. Aqueous solutions of acetic acid and methanol (0.1% v/v) were used as mobile phase with gradient elution; the column temperature was 35°C. Daidzein, glycitein, and genistein (Sigma-Aldrich, Seelze, Germany) standard solutions in methanol were used to identify the products in Buchi (Flawil, Switzerland) samples tested. The isoflavone concentration was calculated by interpolation of the calibration curve prepared by the function of the ratio of the standard area and inner standard area (chrysin) and the ratio of isoflavone and inner standard area in the sample. All experiments were replicated twice.

RESULTS AND DISCUSSION

The compositions of soymilk and cow milk were found to be different with respect to the compounds which could be utilised by the starter and probiotic cultures tested. The parameters of soymilk were as follows: total solids 13.57 ± 0.04% w/w, fat 3.75 ± 0.35% w/w, proteins 2.17 ± 0.02% w/w, and ash 0.61 ± 0.00% w/w, while with cow milk: total solids 12.20 ± 0.05% w/w, fat 3.33 ± 0.29% w/w, proteins 3.59 ± 0.01% w/w, and ash 0.80 ± 0.00% w/w. The pH of soymilk and cow milk were 6.9 and 6.6, respectively. The titratable acidity of soymilk was lower (11.18 ± 0.39 mmol H^+/kg) compared to cow milk (15.85 ± 0.30 mmol H^+/kg).

The variety and quantity of live microorganisms is an important characteristic of the product quality. Cell counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *Bifidobacterium* spp. in cow milk and soymilk after 16 h of fermentation at 37°C are shown in Table 1. At the end of fermentation, no difference in the counts of bifidobacteia fermented alone or in a mixture with the yoghurt culture was observed in either media. The counts of *S. thermophilus* predominated in both media fermented with the yoghurt culture. *L. delbrueckii* subsp. *bulgaricus* achieved counts of 10^7 CFU/ml in milk and of 10^6 CFU/ml in soymilk. The difference could be explained by the inability of *L. delbrueckii* subsp. *bulgaricus* to utilise sucrose, the main sugar in soymilk (Pinthong *et al.* 1980). The same results were found by Donkor *et al.* (2007) who confirmed a better growth of *S. thermophilus* St1342 than that of *L. delbrueckii* subsp. *bulgaricus* Lb1466 in soymilk. A sufficient amount of bifidobacteria in the final product is important in terms of ensuring probiotic properties. In both media, bifidobacteria counts reached more than 10^7 CFU/ml with no difference between the strains used. Several factors influence the growth and viability of *Bifidobacterium* spp. co-cultivated with the yoghurt culture. The acidity, pH and hydrogen peroxide concentration have been identified as having an effects during the manufacture and storage of yoghurt (Lankaputhra *et al.* 1996). On the other hand Farnworth (2007) found that the presence of the probiotic bacteria did not affect the growth of the yoghurt strains in soymilk and cow milk.

The acidification ability has been defined as the difference between the active and titratable acidity measured immediately after inoculation and at the

Table 1. Number of cells (log CFU/ml) in cow milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	Species	Cow milk	Soymilk
YC-381	*S. thermophilus*	8.20 ± 0.12	8.08 ± 0.11
	L. delbrueckii subsp. *bulgaricus*	7.45 ± 0.18	6.46 ± 0.57
YC-381 +BB 12	*S. thermophilus*	8.40 ± 0.45	8.15 ± 0.40
	L. delbrueckii subsp. *bulgaricus*	6.97 ± 0.52	6.38 ± 0.36
	B. animalis subsp. *lactis*	8.36 ± 0.42	8.11 ± 0.18
YC-381 + CCDM 94	*S. thermophilus*	7.28 ± 0.11	8.15 ± 0.12
	L. delbrueckii subsp. *bulgaricus*	7.54 ± 0.19	6.73 ± 0.29
	B. bifidum	8.15 ± 0.21	7.00 ± 0.32
BB 12	*B. animalis* subsp. *lactis*	8.34 ± 0.43	7.08 ± 0.19
CCDM 94	*B. bifidum*	8.15 ± 0.41	7.08 ± 0.64

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Table 2. Changes in pH and titratable acidity (mmol H^+/kg) of cow milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	Cow milk		Soymilk	
	ΔpH16*	ΔTA16*	ΔpH16*	ΔTA16*
YC-381	2.25 ± 0.06	74.0 ± 0.8	2.20 ± 0.14	39.0 ± 0.9
YC-381 + BB 12	2.14 ± 0.15	66.1 ± 0.6	2.25 ± 0.17	46.8 ± 0.7
YC-381 + CCDM 94	2.25 ± 0.10	71.8 ± 0.9	2.27 ± 0.16	46.3 ± 0.7
BB 12	1.60 ± 0.02	38.7 ± 0.4	1.52 ± 0.09	22.8 ± 0.6
CCDM 94	1.05 ± 0.10	17.0 ± 1.2	1.21 ± 0.17	17.5 ± 1.0

*difference in pH or titratable acidity (TA) between 0 and 16 h of fermentation at 37°C

end of fermentation (16 h) at 37°C. The results of all measurements are summarised in Table 2. The addition of bifidobacteria to the yoghurt culture did not influence the pH of either medium but slightly increased the titratable acidity of the soymilk fermented product. The differences in pH between the fermented cow milk and soymilk were not significant (2.2–2.3 in both media). It was assumed that cow milk is a more suitable medium for the acids production. This could be influenced by the fact that starter cultures are adapted to cow milk. Bifidobacteria were only able to acidify the media by half the value compared to the yoghurt culture.

Acetaldehyde and acetic and lactic acids significantly contribute to the products organoleptic quality. The contents of these substances in cow milk and soymilk fermented by all combinations of cultures tested are shown in Table 3. D(–)-Lactic acid is metabolised to a smaller extent than L(+)-lactic acid in the human body and therefore it causes acidosis. Bifidobacteria did not produce D(–)-lactic acid in either medium. Also, Tsangalis and Shah (2004) attempted to quantify D(–) lactic acid in soymilk, they found, however, that none of the strains investigated (*Bifidobacterium pseudolongum* CSCC 1944, *B. longum* CSCC 5550 and CSCC 1941) produced this isomer during the fermentation. Ishibashi and Shimamura (1993) stated that the unique aspect of bifidobacteria is their production of the L(+) form only. Acetic acid is responsible for the undesirable

Table 3. Concentration of lactic acid isomers, acetic acid, and acetaldehyde in cow's milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	L(+)-Lactic acid	D(–)-Lactic acid	Acetic acid	Acetaldehyde (mg/kg)
	(g/100 g)			
Cow milk				
YC-381	0.43 ± 0.00	0.25 ± 0.00	N	5.63 ± 0.36
YC-381 + BB 12	0.41 ± 0.03	0.18 ± 0.01	0.15 ± 0.03	5.25 ± 0.44
YC-381 + CCDM 94	0.39 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	5.96 ± 0.20
BB 12	0.12 ± 0.01	N	0.11 ± 0.00	0.44 ± 0.13
CCDM 94	0.08 ± 0.02	N	0.12 ± 0.01	1.94 ± 0.26
Soymilk				
YC-381	0.29 ± 0.01	0.11 ± 0.01	traces	3.84 ± 0.30
YC-381 + BB 12	0.21 ± 0.01	0.15 ± 0.01	0.03 ± 0.01	4.36 ± 0.39
YC-381 + CCDM 94	0.25 ± 0.01	0.01 ± 0.00	0.12 ± 0.00	6.25 ± 0.33
BB 12	0.08 ± 0.01	N	0.05 ± 0.00	0.37 ± 0.11
CCDM 94	0.12 ± 0.01	N	0.09 ± 0.02	3.59 ± 0.04

N – not detected

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vinegar taste in the products. Both bifidobacteria produced a certain amount of acetic acid. In general, the production of organic acids (sum of lactic and acetic acids) by all organisms in cow milk was higher than in soymilk. Donkor e*t al*. (2007) observed that the selected organisms (*Bifidobacterium lactis* B94 and *B. longum* Bl 536, *L. delbrueckii* subsp. *bulgaricus* Lb 1466 and *S. thermophilus* St 1342) produced lower amounts of organic acids in soymilk than in MRS broth, even if they grew well.

Acetaldehyde is an important volatile flavour compound of yoghurt and is produced by LAB, most frequently from glucose via pyruvate or acetyl phosphate, from amino acids (threonine, methionine, and valine) and from the nucleic acid thymidine. The most important ingredients of cow milk required by the yoghurt starter cultures to produce acetaldehyde are lactose (mainly the glucose fraction) and the amino acids threonine and methionine. The content of acetaldehyde in yoghurt from cow milk usually ranged from 1.3–3.5 mg/kg (Xanthopoulos *et al*. 2001) to 40 mg/kg (Ozer & Atasoy 2002). In this study, milk fermented only by the yoghurt culture contained 5.63 ± 0.36 mg/kg of acetaldehyde while in soymilk yoghurt 3.84 ± 0.30 mg/kg of acetaldehyde was found. This is consistent with the results of Blagden *et al.* (2005) who detected acetaldehyde content of 1.4–3.5 mg/kg in a soy product fermented by *S. thermophilus* after 12 h incubation at 37°C. The production of acetaldehyde by bifidobacteria is also possible. Nosova *et al*. (2000) discovered bifidobacteria strains possessing alcohol dehydrogenase activity, which were capable to oxidise ethanol to acetaldehyde. Margolles and Sanches (2012) found a strain of *Bifidobacterium animalis* subsp. *lactis* CECT 7953 producing acetate, ethanol and yoghurt related volatile compounds (including acetaldehyde) in skim milk. Murti *et al*. (1992) fermented soy extract and cow milk with a yeast extract with *Bifidobacterium* spp. CNRZ 1494, which resulted in the amount of acetaldehyde being higher in the supplemented cow milk (12 ppm) as compared with soy extract (5 ppm). A noteworthy result was obtained with the strain of *B. bifidum* CCDM 94, which produced a significant amount of acetaldehyde in both cow milk and soymilk. In soymilk, the amount of acetaldehyde was almost the same as in the case of the yoghurt culture (3.59 and 3.84 mg/kg, respectively)

Isoflavones can be found in plants as free aglycones, glycosides and malonyl- and acetyl-glycosides. Glycosides belong to the main group of isoflavones which are considered less bioactive. During fermentation, isoflavone glycosides are transformed to aglycons with a higher phytoestrogen activity. Table 4 shows the concentrations of isoflavonoid aglycones in non-fermented soymilk and fermented products. In non-fermented soymilk the bioactive aglycone structure contributes only 8–10% to the total isoflavone content (Tsangalis *et al*. 2003). For example, Ding and Shah (2010) found 0.402 mg/100 ml of total aglycones in soymilk made from soy protein isolate while Chen *et al.* (2010) and Rekha and Vijayalakshmi (2010) who prepared soymilk from soybeans, detected 4.85 mg/100 ml and 2.91 mg/100 ml aglycone forms, respectively. In our samples the total amount of isoflavone aglycones was 1.59 ± 0.06 mg/100 ml in non-fermented soymilk. Only the *B. bifidum* CCDM 94 strain was able to release free aglycons – daidzein and genistein, and therefore to increase their concentrations in the media roughly by a factor of 4. Hydrolysis of β-glycosides by *B. animalis* subsp. *lactis* BB 12 was insignificant and also no aglycons release either was observed after fermentation with the yoghurt culture. The conversion of glycosides to aglycones differed in published data from 0.9–13.9 mg/100 ml in soymilk fermented by probiotic cultures including

Table 4. Concentration of isoflavone aglycones (mg/100 ml) in soymilk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

	Daidzein	Genistein	Glycitein	Sum
Soymilk	0.61 ± 0.02	0.95 ± 0.04	0.03 ± 0.00	1.59 ± 0.06
YC-381	0.50 ± 0.01	0.80 ± 0.02	0.03 ± 0.01	1.32 ± 0.04
YC-381 + BB 12	0.63 ± 0.14	1.08 ± 0.25	0.04 ± 0.01	1.74 ± 0.40
YC-381 + CCDM 94	2.23 ± 0.17	4.02 ± 0.23	0.09 ± 0.01	6.33 ± 0.41
BB 12	0.69 ± 0.04	1.09 ± 0.15	0.02 ± 0.01	1.80 ± 0.21
CCDM 94	2.56 ± 0.74	4.25 ± 1.73	0.10 ± 0.01	6.90 ± 2.47

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bifidobacteria (Pyo *et al.* 2005; Chen *et al.* 2010), however some cultures, for example *Bifidobacterium longum* R0175, were unable to hydrolyse isoflavone glycoside at all (Champagne *et al.* 2010).

CONCLUSION

All three cultures, followed, i.e. the yoghurt culture YC-381 and probiotic cultures (*Bifidobacterium animalis* subsp. *lactis* BB 12, *Bifidobacterium bifidum* CCDM 94) were able to grow in both tested media. *B. animalis* subsp. *lactis* BB 12 is suitable for the production of soy and cow milks fermented products due to a lower level of acetic acid. In soymilk, *B. bifidum* CCDM 94 showed an extensive isoflavone hydrolysis and a higher production of acetaldehyde. The initial counts of LAB and conditions of fermentation were desirable for preparing soy and cow milks fermented products with probiotic microflora.

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