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Biological Stability of Lactofermented Beetroot Juice During Refrigerated Storage

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Beetroot juice fermented by *Lactobacillus brevis* 0944 and *Lactobacillus paracasei* 0920 bacteria was stored for 180 days at 4°C. During storage, samples were taken at 7, 14, 30, 90 and 180 days and the following characteristics were determined: betalain content, ability to limit chemical mutations induced by N-methyl, N'nitro, N- nitrosoguanidine in the Ames test, and the size of the population of *Lactobacillus* bacteria. After 7 days of refrigerated storage of fermented beet juice, betalain content was found to be 88% as against the initial content. For up to 30 days of fermented beet juice storage, betalain content remained at the same level. After 90 and 180 days of storage, the level of betalains falls drastically and amounts to 32% and 25%, respectively, as compared to the initial level. The ability to limit chemical mutations in the Ames test is 57% for *Salmonella* TA 98 and 58% for *Salmonella* TA 100 at the starting point. The antimutagenic activity of fermented beet juice is preserved at the same level for 30 days. After 90 and 180 days, antimutagenic activity decreases to 32% and 41% for *Salmonella* TA 98 and to 41% and 22% for *Salmonella* TA 100. The population of *Lactobacillus* bacteria sp. after fermentation is at a level of 9.11 Log_{10} cfu/mL. After 30 days of storage, the population of bacteria falls to 8.15 Log_{10} cfu/mL. Further storage leads to a decrease in the population of *Lactobacillus* sp. bacteria to 6.80 Log_{10} cfu/mL after 180 days. Fermented beet juice preserves its high biological activity for up to 30 days in refrigerated storage.

INTRODUCTION

Vegetables and fruit are an important source of vitamins, dietary minerals, antioxidative compounds, and dietary fiber. Systematic and substantial consumption of this group of products may prevent chronic non-contagious diseases, popularly referred to as civilization diseases, such as: ischemic heart disease, arterial hypertension, obesity, as well as diet--related cancer [Kanai *et al*., 2006]. The category of health--promoting food currently encompasses products lowering arterial blood pressure, low-fat dairy drinks, probiotics, fruit juices supplemented with calcium, dietary fiber, prebiotics, energy drinks, low-fat cheeses as well as bread enriched with dietary minerals [Urala & Lähteenmäki, 2007]. Particularly promising in creating functional food products are vegetable juices. Their advantages include natural colorants such as carotenoids, chlorophylls, anthocyanins and betalains (betacyanins) capable of neutralizing free radicals responsible for the processes of degradation of cell structures and aberration at the level of DNA [Kapadia, *et al*., 2003; Stintzing & Carle, 2004]. FAO/WHO Expert Consultation [Report of a Joint WHO/FAO, 2003] recommends the consumption of 400–500 g of vegetables and fruit (excluding potatoes) daily. The minimum health-promoting level of vegetable and fruit consumption is considered to be 146 kg/person/year. The most popular vegetables in Europe include cabbage, tomatoes, cucumbers, beetroots, carrots, cauliflower-like vegetables and onions. In the case of beetroots, their share in consumption in Europe amounts to approximately 8% of total vegetables. The beetroot, apart from consumption in its fresh form, is also a valuable vegetable used in the food industry to produce dried and frozen food, non-concentrated and concentrated juices as well as natural colorants (betalains) used as additives in food manufacturing. In many countries there is a growing interest in foods preserved in natural ways. Lactic fermentation is one of the methods of natural preservation and thus production of foods with the highest nutritive value. There are three main types of vegetable juice fermentation: spontaneous fermentation caused by the natural microflora of a particular raw material; fermentation directed by the addition of a starter culture; and controlled fermentation involving the introduction of starter cultures to a thermally-preserved (by pasteurization) raw material [Karovičová & Kohajdová, 2003]. Furthermore, if lactic bacteria with probiotic properties are used in the process of lactic fermentation, a new product will be obtained that may often reveal unique health promoting qualities. The fermented beet juice prepared by the authors contains probiotic *Lactobacillus brevis* 0944 and *Lb. paracasei* 0920 bacteria cultures at the level of 9 Log_{10} cfu/mL. It increases the pool of short chain fatty acids (SCFA), which play a crucial role in nutrition and the proper growth of colonocytes, and decreases the activity of fecal enzymes (β-glucuronidase, β-glucosidase

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and α- and β-galactosidase) responsible for the promotion of neoplastic processes in the intestine [Klewicka *et al*., 2009]. The objective of this work was to determine the stability of betalains, the preservation of biological activity in terms of antimutagenic activity and the survival of *Lactobacillus* sp. bacteria in fermented beet juice during refrigerated storage.

MATERIALS AND METHODS

Plant material

Juice from the Chrobry beetroot variety (*Beta vulgaris* ssp. *vulgaris*) was used in the study. Beetroots for the study were acquired from "Spójnia-Nochowo", Poland. The beetroots were washed, peeled, and cut. The juice was obtained with a yield of 0.7 L/kg using a Bosch MES3000 juice extractor. The juice was pasteurised at 80°C for 10 min.

Microorganisms

Lactic fermentation bacteria were derived from the Collection of Industrial Microorganisms of the Institute of Fermentation Technology and Microbiology ŁOCK 105, Technical University of Lodz. A double strain inoculum consisting in equal volume of *Lactobacillus brevis* 0944 and *Lactobacillus paracasei* 0920 strains was used for fermentation.

Conditions of controlled fermentation

After pasteurization (80°C, 10 min) the juice was cooled down, and inoculum was applied in the amount of 1:10 (v/v). Inoculum was prepared from overnight cultivation of bacteria of the genus *Lactobacillus*. The bacteria biomass was centrifuged at 7840×*g* for 20 min at 4°C. Subsequently, it was suspended in physiological salt solution and adjusted to a density of 7 Log_{10} cfu/mL (each strain separately). The suspensions of the strains were joined at a ratio of 1:1 (v/v), and then thus prepared inoculum was introduced into beet juice. The level of *Lactobacillus* bacteria was 6 Log_{10} cfu/mL of beet juice at the beginning of the fermentation. The process of lactic fermentation of beetroot juice was conducted for 48 h at 30°C.

Storage conditions

After fermentation, the juice was stored in dark glass bottles in a refrigerator at 4°C for 180 days. Samples for assays of betalain content, antimutagenic activity and survival of *Lactobacillus* bacteria were taken at 7, 14, 30, 90 and 180 days.

Spectrophotometric total betalain determination

Betalains content was analysed using Nilsson's spectrophotometric method [Nilsson, 1970]. Samples of the beetroot juice were diluted with a phosphate buffer (pH 6.5) so that the absorbance at 583 nm was between 0.3 and 0.8. Light absorbance value at 583 nm was read on a Beckman spectrophotometer using 1-cm cuvettes.

Quantitative analysis of dye compounds by the HPLC method

A liquid chromatograph ThermoSeparation Product consisting of a Spectra System P2000 pump, photodiode detector UV6000LP, and SN 4000 integrator were used. The separation of pigments was performed on Ace 5 C18 column (250 mm × 4.6 mm i.d.). All samples were filtered through a 0.45 μm Millipore filter prior to chromatography. HPLC method described by Stintzing *et al*. [2002] with a few modifications was used. HPLC conditions were as follows: Eluent A consisted of 2 g/L TFA and 100 g/L HCOOH (65:35, v/v), and eluent B was prepared by mixing 1000 g/L acetonitrile and 100 g/L HCOOH (80:20, v/v). Complete separation of betalains was achieved within 80 min at room temperature and at a flow rate of 0.9 mL/min. The first 15 min were performed isocratically with 100% A, followed by linear gradient from 0 to 20% B in 65 min. Betalains were monitored at 476 and 538 nm for neobetanin and betacyanins, respectively (Table 1). Results were expressed as peak area (PA×10^6). Each set of experiment was repeated twice with triplicate samples. Statistical analyses were performed using STATISTICA program [Czyżowska *et al*., 2006].

TABLE 1. HPLC-DAD data of betalains in beetroot juice.

Name/ trivial name	Retention time (min)	UV-Vis max (nm)
Betanin (betanidin 5-O-β-glucoside)	35.5	538
Isobetanin (isobetanidin 5-O-β-glucoside)	38.3	538
Betanidin	42.8	544
Neobetanidin (14,15-dehydrobetanin)	45.4	476
Isobetanidin	46.2	544

Antimutagenic activity of beetroot juice

The antimutagenic effect of beet juice fermented by bacteria of the genus *Lactobacillus* was determined by the method described by Maron & Ames [1983]. The Ames test used two test cultures of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strains TA 98 and TA 100 (old nomenclature *Salmonella typhimurium)*. In this paper we will use the short name *Salmonella* TA98 or TA100. MNNG (N--methyl, N'nitro, N-nitrosoguanidine) was used as a mutagen in the study (Fluka). MNNG is a strong chemical mutagen not requiring any metabolic activation with the liver fraction S9 for inducing the mutagenic effect [Lankaputhra & Shah, 1998]. MNNG (100 mg/mL) for experiments with *Salmonella* TA 98 and 1 mg/mL for experiments with *Salmonella* TA 100 was dissolved in DMSO (Sigma). Fermented beetroot juice was filtered through filters with a pore diameter of 0.2 mm (Millipore). The prepared juice was added in the amount of 1.0, 2.0 and 10.0 mL/test to 0.1 mL of overnight cultivation of *Salmonella* bacteria of a density of 4.5 × 10^9 cfu/mL and 0.1 mL MNNG (10 mg/plate for TA98 or 0.1 mg/plate for TA100) and incubated at 37 °C for 20 min before adding 2 mL of top agar (ingredient per 100 mL: 0.5 g NaCl, 0.6 g agar). (Before use the top agar was melted by heating the bottle in a steam bath, and 10 mL of a sterile solution of 0.5 mmol/L L-histidine × HCl, 0.5 mmol/L biotin were added to the top agar.) Subsequently, the mixture was deposited on minimal agar (ingredient per 100 mL: $MgSO_4 \times 7\,H_2O$ – 1 g, citric acid monohydrate – 10 g, K_2HPO_4 – 50 g, $Na\,HNH_4PO_4 \times 4H_2O$

– 17.5 g, glucose – 2 g, agar – 1.5 g) pre-prepared on plates. The samples were incubated for 48 h at 37°C without light. Subsequently, the number of *Salmonella* His$^+$ colonies was counted. The spontaneous reversion of His$^+$ mutants for *Salmonella* TA 98 is 36 ± 6 and for *Salmonella* TA 100 is 186±36. The assays were made in three repetitions. Mutation limitation was computed on the basis of the formula:

$$\text{Mutation limitation} = 100 - (N_1 \times 100/N_0)\ [\%];$$

where: N_0 – the number of *Salmonella* His$^+$ colonies (after taking account of spontaneous reversion) determined on plates with MNNG without the addition of beetroot juice; N_1 – the number of *Salmonella* His$^+$ colonies (after taking account of spontaneous reversion) determined on plates with MNNG with an addition of beetroot juice.

Quantity control of bacteria of the genus *Lactobacillus*

The quantity of bacteria of the genus *Lactobacillus* was determined by the plate count method. Juice samples were diluted from 10^{-2} to 10^{-9} in physiological salt solution and poured onto plates. Subsequently, MRS Agar medium (BTL sp. z o.o., Poland) was poured onto the plates, and then the plates were incubated at 30°C for 48–72 h. Subsequently, the plates were counted. The results are presented as Log_{10} cfu/mL.

Statistical analysis

Statistical analysis of the results was made with the use of STATISTICA program, OriginPro version 7.5 and Microsoft Excel 2000. Statistical differences were computed with the use of the One-way ANOVA test.

RESULTS AND DISCUSSION

Betalain stability

In fresh beet juice, the prevailing red colorants (betacyanins) are betanin and isobetanin, whose content is estimated to be approximately 80 to 90%, as well as neobetanin, whose content is much lower at about 6% [Czyżowska *et al.*, 2006; Stintzing *et al*., 2006]. Betanin and isobetanin contain phenolic and acylic amine groups, which are thought to impart high antioxidative potential [Frank *et al*., 2005]. The research team under the direction of Czapski investigated the antioxidant value of the juice extracted from 11 varieties of red beet: Ceryl, Chrobry, Czerwona Kula, Nochowski, Noe, 21, Noe 694, Noe 904, Noe Pol, Okrągły Ciemnoczerwony, Opolski, Wodan. The antioxidant value of these juices was at the level from 10.2 to 20.6 Trolox μmol/mL [Czapski *et al*., 2009].

Beetroots are consumed (especially in Central and Eastern Europe) raw, cooked as a vegetable, and also in the form of food products such as lacto-fermented juice, concentrated juice and pickled preserves. Betalains content in fresh beetroot juice is at a level of 1.27 mg/mL, whereas after pasteurization betalains concentration in the juice is reduced to 1.01 mg/mL (Table 2). Beetroot juice fermented by selected *Lactobacillus brevis* 0944 and *Lactobacillus paracasei* 0920 strains is characterised by a high content of betanin and isobetanin, 352×10^6 (76% of total red colorant content) and 67×10^6 (peak area), which constitutes 14% of the total content of red colorants. The content of neobetanin remains at a level of about 3% of total betalain content. Apart from betanin, isobetanin, and neobetanin, the fermented juice contains: betanidin (5% of total red dye content) and isobetanidin (0.7% of total red colorant content). These compounds are not found in fresh beet juices, which was shown in a study by Czyżowska *et al*. [2006]. Betanidine and isobetanidine are aglycones, which are thought to have high biological activity (just as betanin) with respect to neutralizing free radicals present in the environment [Kanner *et al*., 2001]. Betanidin and its isomer – isobetanidin – are formed in fermented beet juice as a result of the bacterial activity of β-glucosidase catalysing the transformation of betanin into betanidine [Stintzing & Carle, 2004]. During the storage of fermented beet juice, at 7 days a decrease in the content of betanin by 17%, isobetanin by 4% and betanidin by 17% was observed. Isobetanidin content remained unchanged at 7 days of storage (Table 2). In the case of neobetanidin, after seven days of juice storage, a 54% increase in this compound content was found. After 30 days of fermented beetroot juice storage, betanin content did not change with respect to its content at 7 days. The concentration of isobetanin decreased by 5% as against its level at 7 days of storage and by 9% as

TABLE 2. Total content and qualitative composition of betalains in fresh and fermented beetroot juice (peak area × 10^6±SD) during storage for 0–6 months at 4°C.

Storage time of fermented juice (day)	Betalains content (mg/mL)	Betanin	Isobetanin	Betanidin	Isobetanidin	Neobetanin
0	0.96±0.002	352±3.56	67±0.52	23±3.50	3±0.01	14±0.12
7	(0.57±0.002)*	295±0.23	64±0.63	20±0.36	3±0.10	(22±0.36)*
14	(0.58±0.001)*	275±0.36	64±2.30	14±0.36	3±0.02	(22±0.52)*
30	(0.64±0.006)*	298±2.36	61±0.25	13±0.12	(2±0.20)*	(37±0.25)*
90	(0.21±0.002)*	(77±0.26)*	(32±0.28)*	S	S	(38±0.34)*
180	(0.31±0.003)*	(50±3.25)*	(26±0.25)*	S	S	(38±0.13)*
Fresh juice	1.27±0.008	405±6.28	134±2.08	S	S	31±0.23
Juice after pasteurization	1.01±0.005	368±7.23	92±1.86	S	S	22±0.12

Average value n=3, SD – standard deviation, S – trace, * result statistically significant in the Anova test, p≤0.05, with respect to the starting value determined at the starting point.

against the starting point. Betanidin and isobetanidin content after 30 days decreased by 45% and 25%, respectively, with respect to the starting point. Neobetanidin content increased by 149% with respect to the starting point at 30 days of storage. During further refrigerated storage of fermented beet juice, that is at 90 and 180 days, a dramatic fall in betanin was observed, by 78% and 86%, respectively, as well as a drop in isobetanin by 52% and 60%, respectively, as compared to the starting point. Betanidin and isobetanidin content at 90 and 180 days of juice storage was negligible. Neobetanin concentration during storage of over 30 days increased by 159% after 90 days, and by 157% after 180 days, as compared to the starting point. The recorded increase in neobetanin content during fermented beet juice storage results from the processes that betanin undergoes in low pH [Stintzing & Carle, 2004]. Beetroot juice after fermentation by *Lb. brevis* 0944 and *Lb. paracasei* 0920 bacteria is characterised by a pH of 3.9 [Czyżowska *et al*., 2006]. Studies conducted by Stintzing *et al.* [2006] revealed that betalains were stable during storage for 22 days at 94% in a pH of 4.5 and 77% in a pH of 3.5. Other authors, during storage of pasteurised beet pulp during 60 days at 5°C, recorded a 7% drop in betanin content in the stored substance [Pátkai *et al*., 1997]. In the presented study, we found 89% stability of red colorants in fermented beet juice during 30-day refrigerated storage.

Suppression of chemical mutations

The ability to limit chemical mutations was determined for fermented beetroot juices in refrigerated storage by the Ames test with respect to *Salmonella* TA 98 and TA 100. Antimutagenic properties of fresh and fermented beetroot juice have been described by Klewicka [2010]. Fresh beetroot juice used in a dose of 10 μL/test reduces the formation of mutations induced by MNNG from 64 to 65%. After spontaneous fermentation of the beetroot juice reduction of mutation ranges from 24% to 28%. Beetroot juice after controlled fermentation and has the ability to reduce the level of mutation from 50 to 65% for 10 μL/test dose, depending on the used starter [Klewicka, 2010]. At the starting point of storage, fermented juice was characterised by the ability to limit chemical mutations induced by means of MNNG by 57% for *Salmonella* TA 98 (at a dose of juice of 10 mL/test) and by 58% for *Salmonella* TA 100 (at a dose of juice of 10 mL/test) (Table 3). After 7 days of refrigerated storage of fermented beetroot juice, the ability to limit chemical mutations decreased to 54% for both *Salmonella* strains. After 30 days of refrigerated storage of our product, suppression of chemical mutations still remained at a high level – 57% in the case of *Salmonella* TA 98 and 62% for *Salmonella* TA 100 (at a dose of juice of 10 mL/test). After 90 and 180 days of juice storage, antimutagenic activity was decreased for *Salmonella* TA 98 to 32% and 10%, respectively, while for *Salmonella* TA 100 to 41% and 22%, respectively. The storage of fermented beetroot juice for over 30 days results in a dramatic decrease in its ability to limit the occurrence of chemical mutations. This is related to the stability of red dyes during refrigeration. The biological activity of beetroot juice, including its anticancer and antimutagenic

TABLE 3. Antimutagenic properties of fermented beetroot juice in the Ames test with the use of *Salmonella* TA 98 and TA 100 with MNNG (10 mg/test) during storage for 0–6 months at 4°C.

Storage time (day)	Juice dose (mL/test)	*Salmonella* TA 98		*Salmonella* TA 100	
		Mutants His^+/test[a]±SD	Mutation limitation (%)	Mutants His^+/test[a]±SD	Mutation limitation (%)
0	0.0	141±24	–	1789±172	–
	2.0	(92±8)*	34	(1055±98)*	41
	10.0	(60±12)*	57	(736±64)*	58
7	0.0	139±26	–	1891±162	–
	2.0	100±15	28	(1201±101)*	36
	10.0	(63±8)*	54	(853±25)*	54
14	0.0	148±25	–	1715±130	–
	2.0	(93±10)*	37	(1123±134)*	34
	10.0	(72±10)*	51	(714±56)*	58
30	0.0	151±14	–	1986±163	–
	2.0	(95±8)*	37	(1385±145)*	30
	10.0	(64±9)*	57	(752±42)*	62
90	0.0	148±16	–	1769±142	–
	2.0	114±15	22	1536±123	13
	10.0	100±4	32	(1041±63)*	41
180	0.0	143±29	–	1799±143	–
	2.0	131±8	8	1520±186	15
	10.0	128±19	10	1398±75	22

[a] Average value n=3; SD – standard deviation, * Statistically significant difference with respect to the control value (without juice), Anova test, $p \leq 0.05$.

properties, are attributed by many authors to the colorant compounds present in the root of beetroots, that is, betacyanins (betanin, isobetanin, and other related compounds) [Haveland-Smith, 1981; Kapadia *et al*., 2003; Strack *et al*., 2003; Stintzing & Carle, 2004]. In our study, we have found that these colorants are stable for up to 30 days of refrigerated storage, and after this time the amount of the colorants declines and consequently the antimutagenic properties of fermented beet juice deteriorate.

Survival of *Lactobacillus* bacteria

Six logarithmic units/mL of cells of *Lactobacillus* bacteria were added to fresh beetroot juice. After 48-h fermentation at 30°C, the total number of live *Lactobacillus* sp. cells was determined (without counting particular species). After lactic fermentation conducted in beetroot juice, the number of bacteria cells was found to increase from 6 logarithmic units to 9.11 Log_{10} cfu/mL (an increase by 3 Log_{10} cfu/mL). During refrigerated storage, after 7 days there is a decrease in the number of bacteria of the genus *Lactobacillus* by 0.13 Log_{10} cfu/mL, and after 14 days by 0.67 Log_{10} cfu/mL. After 30 days of storage, the number of the bacteria was 8.15 Log_{10} cfu/mL (-0.85 Log_{10} cfu/mL relative to the starting point). During further storage of fermented beet juice, there was a tendency for the bacteria population to decrease: at 90 days to 7.40 logarithmic units and at 180 days to 6.80 Log_{10} cfu/mL (Table 4). The factor critical to food containing live microorganisms is the preservation of their adequate numbers in the product throughout its shelf life. For probiotic bacteria strains, it is thought that the minimum is 6 Log_{10} cfu/mL per g of product or 9 Log_{10} cfu/mL per single dose, *e.g.* capsule [Reid *et al*., 2001]. Yoon *et al.* [2005] stored beet juice fermented by various lactic bacteria species for 4 weeks. These authors used the following strains for fermentation: *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum* and *Lb. debrueckii*. *Lb. casei*, *Lb. plantarum* and *Lb. debrueckii* cultures were characterised by a high survival rate during storage (6–7 Log_{10} cfu/mL). In beet juice fermented by *Lb. brevis* 0944 and *Lb. paracasei* 0920 bacteria, the quantity of these microorganisms meets the criteria for products containing live microorganisms throughout storage (180 days), and their quantity in the product is 6.80 Log_{10} cfu/mL.

TABLE 4. *Lactobacillus* bacteria survival in fermented beetroot juice during storage for 0–6 months at 4°C.

Storage time	Fermented juice
(day)	Number of live *Lactobacillus* sp. cells (Log cfu/mL[a] ±SD)
0	9.11±0.30
7	8.98±0.55
14	8.44±0.86
30	8.15±0.75
90	(7.40±0.81)*
180	(6.80±0.98)*

[a] Average value n=3; SD – standard deviation, * Statistically significant difference with respect to the control value (storage time, 0 day), ANOVA test, p≤0.05.

CONCLUSIONS

Fermented beet juice containing live bacteria of the genus *Lactobacillus* meets the criteria specified for functional products. Functional products are defined as food products made from natural raw materials with proven health promoting properties. Fermented beetroot juice both reveals the advantages of plant-based products, and in particular the biological activity of betalains, as well as the probiotic qualities resulting from the presence of live bacteria with such qualities. It is very important for every functional product to determine a maximum storage time, during which the product preserves its bioactive properties. For our product, this time is 30 days at a temperature of 4°C. An additional advantage of fermented beetroot juice is the absence of chemical preservatives. The role of substances suppressing the growth of microflora contaminating food is played by lactic acid and acetic acid synthesised during lactic fermentation from saccharides present in beet juice.

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