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Fermented Beetroot Juice as a Factor Limiting Chemical Mutations Induced by MNNG in *Salmonella typhimurium* TA98 and TA100 Strains

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Summary

The ability of fresh and fermented beetroot juice to limit chemical mutations has been studied using the Ames test and the strains of *Salmonella typhimurium* TA98 and TA100. Beetroot juice was fermented in either spontaneous or controlled fermentation, with the use of selected cultures of lactic acid bacteria, three *Lactobacillus paracasei* strains designated as 0916, 0920, 0923, and one *Lactobacillus brevis* strain 0944. *N*-methyl-*N'*-nitro-*N*--nitrosoguanidine (MNNG) was used as a standard mutagen for the induction of His^+ revertants in mutations of *Salmonella typhimurium* TA98 and TA100. The ability to reduce mutations was studied using the Ames test and the doses of beetroot juice of 0.5, 1.0, 2.0, 5.0 and 10.0 μL per plate. The study showed that the fermented beetroot juice (10 μL/plate) reduced the level of MNNG-induced mutations by 64 % in *Salmonella typhimurium* TA98 and by 65 % in *Salmonella typhimurium* TA100. The beetroot juice obtained by spontaneous fermentation retained only 24–25 % of initial antimutagenic activity (in *Salmonella* strain and at the highest tested dose of the juice, *i.e.* 10 μL/plate). The doses of 10 μL/plate of the beetroot juice fermented by three *L. paracasei* cultures (0916, 0920 and 0923) decreased the intensity of mutations induced by MNNG by 61, 50 and 56 % in *Salmonella typhimurium* TA98 and by 65, 56 and 49 % in *Salmonella typhimurium* TA100, respectively. The juice (10 μL/plate) fermented by *L. brevis* 0944 strain reduced the number of mutations by 58 % in *Salmonella typhimurium* TA98 and by 55 % in *Salmonella typhimurium* TA100. Thus, the controlled lactic acid fermentation of beetroot juice conducted by selected *Lactobacillus* strains maintains its antimutagenic activity.

Key words: fermented juice, beetroot, *Salmonella typhimurium*, *Lactobacillus*, antimutagenic activity

Introduction

Studies into natural and synthetic chemoprotectants effective in the prophylaxis and supportive treatment of cancer, preventing mutagenesis that leads to negative changes at the level of DNA (*1*), revealed the high protective capacity of betanin (*2–4*). The richest source of this compound and other betalains is beetroot (*Beta vulgaris* L.). It is a popular vegetable in human diet. Red beetroot pigments do not induce any chromosome aberrations in guinea pig fibroblasts (*5*). It is thought that the antioxidative and anticarcinogenic activity of beetroot is due to betalains, and in particular betanin and isobetanin. Their share is 75 to even 95 % of betanin with respect to the general content of betacyanins, while the share of isobetanin is much lower at 20 % maximum (*4,6*). Betalains are water-soluble pigments. Their stability during heating and storage is weak and depends on pH (they are most stable at pH between 4 and 6) (*6*).

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The dietary factors that protect from diet-dependent cancers are believed to be vegetables, fruit and other plant foods that are rich in dietary fibre (*7*). WHO/FAO experts (*8*) recommend consuming 400–500 g of vegetables and fruit (without potatoes) a day in order to reduce the risk of heart disease, cancer, diabetes, and obesity. Red beetroot is a popular vegetable in Europe and is widely consumed in Germany, France and the Mediterranean countries. In Poland, red beetroot has been grown since the 16th century and became even more popular in the 20th century. Currently, in Poland red beetroot is grown in the area of 20–22 000 ha, and ranks fourth (after cabbage, carrot and onion) in terms of the crop (450–550 000 t). Poland is the leading beetroot producer in the European Union. Red beetroot is also used in manufacturing of dried and frozen foods, juices and their concentrates, and as natural pigments (betalains) used as additives in food manufacturing. In Poland, the red beetroot juice has also been subjected to lactic acid fermentation.

Fermentation of beetroot juice requires selected starter cultures, like lactic acid bacteria (LAB), which also populate this vegetable but their number is usually very small. Therefore, they are incapable of conducting lactic acid fermentation themselves and do not ensure satisfying sensory properties of the fermented juice. Production of fermented red beetroot juice with high nutritional value, good stability and antimutagenic activity requires application of appropriate starter cultures of lactic acid bacteria. The additional advantage of the fermented product is the presence of probiotic microflora, which improves the health promoting activity. An objective of this study is to obtain the fermented red beetroot juice displaying high biological activity with the focus on its antimutagenic potential.

Materials and Methods

Plant material

Juice from the beetroot (*Beta vulgaris* L. var. Chrobry) was used in the study. Beetroot was purchased from Spójnia-Nochowo Ltd, Nochowo, Poland. The vegetables were washed, peeled, and sliced. The juice produced at a yield of approx. 0.8 L/kg was frozen and stored at –20 °C. Before fermentation, the juice was thawed at room temperature.

Microorganisms

Lactic acid bacteria originated from the Collection of Industrial Microorganisms at the Institute of Fermentation Technology and Microbiology (ŁOCK 105), Lodz, Poland. Three strains of *Lactobacillus paracasei* (0916, 0920 and 0923) were isolated from human intestinal tract while *Lactobacillus brevis* 0944 was isolated from a plant.

Conditions of controlled fermentation

The thawed beetroot juice was pasteurized at 80 °C for 10 min, cooled and inoculated (10 % by volume) with *Lactobacillus* bacteria that were grown overnight, harvested by centrifugation (10 000 rpm, 20 min, 4 °C) and suspended in the physiological saline (cell density of 10^6 CFU/mL). The lactic acid fermentation of beetroot juice was conducted for 48 h at 30 °C. Then, the juice was filtered through 0.2-μm membranes (Millipore, Ireland).

Conditions of spontaneous fermentation

The thawed beetroot juice (without pasteurization) was incubated at 30 °C for 5 days. The spontaneous fermentation lasted for as much as 5 days because the initial number of *Lactobacillus* cells (10^2–10^3 CFU/mL) populating this juice was too low to ferment it earlier. After the fermentation was complete, the juice was filtered through 0.2-μm membranes (Millipore, Ireland).

Antimutagenic activity of beetroot juice

The antimutagenic effect of the raw beetroot juice and the beetroot juice fermented by the selected strains of the genus *Lactobacillus* was determined using the method described by Maron and Ames (*9*). The Ames test used two cultures of *Salmonella typhimurium* designated as TA98 and TA100 (*10*). In this study, MNNG (Fluka, Switzerland) was used as a direct-acting mutagen. It does not require any metabolic activation with the liver fraction S9 to induce the mutagenic effect (*11*). The concentration of MNNG solution in DMSO (Sigma, USA) was 100 μg/mL for experiments with *Salmonella typhimurium* TA98 and 1 μg/mL for experiments with *Salmonella typhimurium* TA100. These doses of mutagen were determined on the basis of experimental curves presenting the dependence of the quantity of His^+ revertants on the mutagen concentration. For *Salmonella typhimurium* TA98 and TA100, the applied mutagen doses ensured the appearance of 200 and 2000 colonies of His^+ revertants, respectively. Samples of fresh and fermented beetroot juices were filtered through 0.2-μm membranes (Millipore, Ireland). Their doses of 0.5, 1.0, 2.0, 5.0 and 10.0 μL/plate were mixed with 0.1 mL of the samples of overnight cultures of *Salmonella typhimurium* bacteria (cell density of $4.5 \cdot 10^9$ CFU/mL) and 0.1 mL aliquots of MNNG (10 μg/plate for TA98 or 0.1 μg/plate for TA100) and incubated at 37 °C for 20 min before adding 2 mL of top agar. Then, these mixtures were spread on the minimal essential agar plates. The plates were incubated for 48 h at 37 °C in darkness prior to counting of *Salmonella* His^+ colonies. All samples were prepared in triplicate. A decrease in the number of mutations was computed from the equation:

$$\text{Mutation reduction} = 100 - (N_1 \times 100 / N_0)/\% \qquad /1/$$

where N_0 is the number of *Salmonella* His^+ colonies (the number of spontaneous revertants was taken into consideration) visible on the plates containing samples with MNNG but without the beetroot juice, and N_1 is the number of *Salmonella* His^+ colonies (the number of spontaneous revertants was taken into consideration) visible on the plates containing samples supplemented with MNNG and beetroot juice. The results take into account the spontaneous reversion. In *Salmonella* TA98 the spontaneous reversion of His^+ mutants was 33±7 and for *Salmonella* TA100 it was 198±32. In *Salmonella typhimurium* TA98 the MNNG concentration was 10.0 μg/plate and in *Salmonella typhimurium* TA100 it was 0.1 μg/plate.

Enzymatic assays of lactic and acetic acids

Lactic and acetic acids were quantified by enzymatic methods using commercial kits purchased from Boehringer Mannheim/R-Biopharm (Manheim, Germany).

Statistical analysis

The experimental data were expressed as mean± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was applied to find the difference between the bacterial populations treated with mutagen and with mutagen and beetroot juice (at $p<0.05$).

Results and Discussion

The antimutagenic activity of nonfermented and fermented beetroot juices in *Salmonella typhimurium* TA98 and TA100 was determined by the Ames test. Fresh red beetroot juice reduced the intensity of mutations induced by MNNG in a dose-dependent manner by 15–65 % in *Salmonella typhimurium* TA98 and by 21–64 % in *Salmonella typhimurium* TA100 (Table 1). The beetroot juice was subjected to spontaneous and controlled lactic acid fermentation to determine their effect on its antimutagenic activity. The spontaneous fermentation of beetroot juice considerably decreased this activity (by 2.5-fold compared to the fresh juice). The number of mutations was decreased in a dose-dependent manner, by 7.5–24 % for *Salmonella typhimurium* TA98 and by 5–28 % in *Salmonella typhimurium* TA100 (Table 1). The spontaneously fermented juice was characterized by a soil-like taste and aroma. Its colour was changed from dark maroon to brown. Both the taste and aroma were evaluated as unacceptable.

The selected cultures of lactic acid bacteria were used in the controlled red beetroot juice fermentation, which ensured acceptable sensory properties like taste, colour, and aroma. The juice (a dose of 10 μL/plate) fermented with *Lactobacillus paracasei* 0916 strain decreased the number of mutations of *Salmonella typhimurium* TA98 and *S. typhimurium* TA100 by 61 and 65 %, respectively (Table 2), like the fresh beetroot juice. When the juice was fermented by *L. paracasei* 0920, its dose of 10 μL/plate reduced mutations of *Salmonella typhimurium* TA98 and *S. typhimurium* TA100 by 50 and 56 %, respectively (Table 2).

Table 1. Antimutagenic activity of fresh and spontaneously fermented beetroot juice in *Salmonella typhimurium* TA98 and *S. typhimurium* TA100 determined by the Ames test

V(juice) / μL/plate	Fresh beetroot juice				Spontaneous fermentation of beetroot juice			
	TA 98		TA100		TA98		TA100	
	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %
0.0	132±16	–	1679±152	–	132±16	–	1679±152	–
0.5	112±2	15	(1325±56)*	21	122±22	7.5	1592±133	5
1.0	(95±6)*	28	(1069±56)*	36	(115±16)*	12.0	1452±102	8
2.0	(85±3)*	35	(951±65)*	43	(100±13)*	24.0	(1212±105)*	27
5.0	(69±9)*	47	(851±26)*	49	(101±12)*	23.0	(1200±35)*	28
10.0	(45±6)*	65	(589±100)*	64	(100±15)*	24.0	(1201±56)*	28

Number of analyzed plates $N=3$; *statistically significant difference compared to the control value (without juice), ANOVA test ($p<0.05$)

Table 2. Antimutagenic activity of beetroot juice fermented by *Lactobacillus paracasei* 0916 and *L. paracasei* 0920 in *Salmonella typhimurium* TA98 and *S. typhimurium* TA100 determined by the Ames test

V(juice) / μL/plate	*Lactobacillus paracasei* 0916				*Lactobacillus paracasei* 0920			
	TA98		TA100		TA98		TA100	
	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %
0.0	132±16	–	1679±152	–	132±16	–	1679±152	–
0.5	102±15	23	1351±56	20	128±25	4	1536±58	9
1.0	(95±12)*	29	(1002±100)*	41	112±12	16	1320±102	21
2.0	(89±8)*	33	(975±86)*	42	(85±8)*	36	(1009±56)*	40
5.0	(75±3)*	44	(795±23)*	53	(78±4)*	40	(899±45)*	46
10.0	(52±13)*	61	(602±45)*	65	(65±12)*	50	(736±96)*	56

Number of analyzed plates $N=3$; *statistically significant difference compared to the control value (without juice), ANOVA test ($p<0.05$)

The red beetroot juice fermented by *L. paracasei* 0923 (a dose of 10 μL/plate) also lowered the number of mutations induced by MNNG (by 56 % in *S. typhimurium* TA98 and by 49 % in *S. typhimurium* TA100 (Table 3).

Also, the application of *L. brevis* 0944 in red beetroot juice fermentation retained its ability to reduce mutations of *Salmonella typhimurium* TA98 (by 56 %) and *S. typhimurium* TA100 (by 55 %), at a dose of 10 μL/plate (Table 3). Thus, the red beetroot juice fermented by the tested strains of lactic bacteria (*Lactobacillus* spp. 0916, 0920, 0923 and 0944) prevented mutations caused by MNNG, the same as the fresh juice. The biological activity of beetroot juice, like anticancer and antimutagenic properties, is ascribed by many authors to the pigments present in the root of beetroot, like betacyanins (betanin, isobetanin, and other related compounds) (*1,4,6,12*). Concentration of betalains before and after lactic acid fermentation of the red beetroot juice was determined by Czyżowska *et al.* (*13*). These investigations revealed that the spontaneous fermentation decreased the content of total betalains (betanin, isobetanin and neobetanidin) by as much as 97 % (*13*). Therefore, this juice showed relatively low activity in reducing mutations at a level of 24–28 % (for the largest tested dose of 10 μL/plate). By contrast, the controlled lactic acid fermentation of beetroot juice decreased the total content of betalains only by 17 to 47 %, depending on the strain. The same authors found that the controlled lactic acid fermentation changed the profile of red pigments. In nonfermented juice, the dominating pigments are betanin and isobetanin. The controlled lactic fermentation of beetroot juice by bacteria of the genus *Lactobacillus* drastically decreases the amount of betanin and isobetanin, and liberates their aglycones betanidine and isobetanidine (*13*). These aglycones also display high biological activity (*6*). In betacyanins, glycosylation reduces the biological activity, while acylation generally increases the antioxidant potential. The glycosylated form (betanin) dominates in the fresh red beetroot juice. Organic acids and β-glucosidase synthesized by bacteria conducting the controlled lactic acid fermentation convert this form to betanidin and isonetanidin (aglycones). The higher biological activity of betanidin and isobetanidin results from their susceptibility to acylation at 5-O- and 6-O-positions (*6,12*). Despite the change in the quantitative and qualitative composition of betalains in juice obtained in the controlled lactic acid fermentation, its antimutagenic activity is almost the same as that of the fresh juice.

When the fermentation is complete, the red beetroot juice also contains various metabolites of lactic acid bacteria like organic acids (lactic and acetic), which display antimutagenic properties (*11*). Thus, the maintenance of mutation-limiting properties after fermentation may be explained by the presence of the acidic metabolites produced by lactic bacteria. The lactic bacteria used in this study are characterized by the relatively heterofermentative metabolism (*L. paracasei*) or strictly heterofermentative metabolism (*L. brevis*). As a result of fermentation of the saccharides contained in the beetroot juice, these bacteria form a mixture of organic acids rich in lactic (8.0 to 11.0 g/L, the main component) and acetic (0.2 to 4.6 g/L) acids (Table 4).

Table 4. Lactic and acetic acid concentrations in the fermented beetroot juice±S.D. (*N*=3)

Strain	γ(g/L)	
	LA	AA
L. paracasei 0916	10.40±0.17	4.47±0.42
L. paracasei 0920	10.40±0.24	0.89±0.07
L. paracasei 0923	8.10±0.14	5.21±0.24
L. brevis 0944	9.01±0.01	4.58±0.71
Spontaneous fermentation	5.50±0.05	0.20±0.01

LA – lactic acid, AA – acetic acid; S.D. – standard deviation

The quantity of lactic and acetic acids produced by the spontaneous fermentation of the red beetroot juice was much lower (5.50 and 0.2 g/L, respectively). Using the Ames test, Lankaputhra and Shah (*11*) found that acetic acid displayed higher antimutagenic activity than lactic acid (for MNNG). The capability of reducing the mutagenic potential of MNNG by acetic acid (in Ames test) can be explained by the adaptive cytoprotection. Low concentrations of acetic acid (1 %) are thought to tough-

Table 3. Antimutagenic activity of beetroot juice fermented by *Lactobacillus paracasei* 00923 and *L. brevis* 0944 in *Salmonella typhimurium* TA98 and *S. typhimurium* TA100 determined by the Ames test

V(juice) / μL/plate	*Lactobacillus paracasei* 0923				*Lactobacillus brevis* 0944			
	TA98		TA100		TA98		TA100	
	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %	His^+ mutants plate±S.D.	Mutation reduction %
0.0	132±16	–	1679±152	–	132±16	–	1679±152	–
0.5	121±23	8	1621±32	3	102±12	22	1368±25	18
1.0	(100±15)*	25	1359±102	19	(95±8)*	28	(1106±56)*	34
2.0	(82±6)*	37	(1123±95)*	33	(81±6)*	38	(912±36)*	45
5.0	(75±6)*	43	(915±65)*	45	(70±6)*	46	(856±98)*	49
10.0	(58±7)*	56	(856±63)*	49	(58±4)*	56	(754±58)*	55

Number of analyzed plates *N*=3; *statistically significant difference compared to the control value (without juice), ANOVA test ($p<0.05$)

en intestinal endothelial cells or bacteria and, as a consequence, they are more resistant to higher concentrations of acetic acid or other toxic compounds (*14*). Bacterial strains used in this study for fermentation of red beetroot juice synthesize 0.89 to 5.21 g/L of acetic acid. Because of the antimutagenic activity of acetic acid, heterofermentative strains should be used in production of fermented foods.

Conclusions

In conclusion, the present study provides evidence that the controlled lactic acid fermentation of beetroot juice maintains its antimutagenic activity. Factors determining this activity are betalains and certain products of lactic acid bacteria metabolism, like acetic acid.

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