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Original article
Red Beetroot Thematic Issue

Bioactive Compounds and Microbial Quality of Stored Fermented Red Beetroots and Red Beetroot Juice

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The aim of this study was to investigate the effect of long-term cold storage of fermented beetroots and fermented beetroot juice on the content of biologically active compounds and microorganisms. Contents of total phenolics, as well as red and yellow betalains were determined. Total phenolics content in fermented grated beetroot was 920 mg/kg after 7 months of cold storage, while in juice it was 810 mg/L within the same timespan. At the end of the experiment, after 10 months of storage, these values decreased to 570 mg/kg and 540 mg/L, respectively. Concentration of red betalains after 7 months of storage was determined at 116 mg/kg for grated beetroot and at 69 mg/L for juice. At the same time, the content of yellow betalains was 14 mg/kg and 19 mg/L for grated beetroot and juice, respectively. In the case of fermented beets and juice, about 3-fold decrease of red pigments was observed during storage. Bioactive compounds were identified using LC-MS. Betanidin was shown to be the major compound among grated beetroot pigments at the beginning of the investigation, the beetroot juice was predominated by isobetanidin and betanidin. At the end of the study, the proportion of pigments changed slightly. Lactic acid bacteria predominated among the bacterial microbiota in the products tested. *Enterobacteriaceae* were not detected in fermented grated beetroots and investigated juices throughout storage time. To conclude, during long-term cold storage, the content of bioactive compounds decreases, however, remains at a high level.

INTRODUCTION

The preservation of plant products by fermentation was known in ancient times, mainly in the Far East. This method was widely used in countries engaged in seasonal plant cultivation. It also made it possible to secure the food in the event of natural disasters. Fermentation is one of the oldest technologies used until today, which prolongs the shelf life of food products and increases their nutritional and organoleptic values [Kavitake *et al*., 2018]. The tradition of natural food preservation is related to the geographical area, climate, and the type of ingredients used. Fermented food is widely used in East Asia; fermenting vegetables and fruits is very important in the northern European countries, while the production of fermented olives and fermented sausages dominates in the southern Europe. Fermentation of cucumbers, cabbage, and olives plays also the most important economic role. Fermented: red beetroots, cauliflower, carrot, celery, onion, pepper, and tomatoes are less known.

The fermentation process affords the opportunity to obtain a large range of new products that will differ in their organoleptic characteristics from the primary raw material. The process of fermentation and the beneficial effect of fermented products on our organism [Swain *et al.*, 2014] causes their popularity and increases a group of supporters. Lactic acid from kimchi may prevent fat accumulation and obesity-induced heart diseases [Park *et al.*, 2008]. Kimchi has been reported to have anti-obesity effects [Jung *et al.*, 2014; Kim *et al.*, 2011; Park *et al.,* 2012]. Some studies have documented the protective effects of sauerkraut or sauerkraut juice against breast [Ju *et al.*, 2000; Licznerska *et al.*, 2013; Szaefer *et al.*, 2015] and colon [Kusznierewicz *et al.,* 2010] cancer. Microorganisms involved in the fermentation process (lactic acid bacteria like: *Lactobacillus*, *Leuconostoc*, and *Streptococcus* genera) impart the products a characteristic sour taste, form aromatic compounds [McFeeters, 2004], change the structure of fermented plant material [Parada & Aguilera, 2007], increase the digestibility of the plant mass [Swain *et al.*, 2014] as well as increase the content of vitamins (mainly B2 and PP ones) [Capozzi *et al.,* 2012; Thapa & Tamang, 2015]. Other functions of microorganisms include elimination of undesirable substances that can be found in plant raw materials (cyanides, gas-forming substances, hemagglutinins, thioglycosides) and bioconservation, *i.e.* protection against the development of undesirable microbiota [Septembre-Malaterre *et al.,* 2018].

Harmful microorganisms that may occur in improperly prepared fermented products include: putrefactive bacteria – aerobic and anaerobic ones, having proteolytic properties responsible for food spoilage; butyric acid bacteria, cellulo-

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lytic and pectinolytic bacteria – violating the structure of plant tissues [Zieliński *et al*., 2017], which results in "smeariness"; and bacteria of false lactic fermentation, producing carbon dioxide and unwanted products having the nature of organic acids [Franco *et al.*, 2012]. A significant threat can also be yeasts and molds, the development of which is harmful to products of fermentation [Perez Diaz *et al.*, 2014]. In addition, molds can produce toxic substances, breakdown plant tissues [Maruvada & McFeeters, 2009], and can also cause secondary acidification of the environment leading to the development of putrefactive bacteria [Franco *et al.*, 2012]. On the other hand, a small amount of yeast is favorable, as it stabilizes the microbiota [Arroyo-Lopez *et al*., 2012]. It is the ethanol produced by yeast that acts as a preservative. In addition, yeast produces vitamins and growth substances that favorably affect lactic acid bacteria [Arroyo-Lopez *et al.,* 2012]. However, when excessively growing in the fermented product, yeast become the prevailing microbiota, competing with lactic acid bacteria [Arroyo-Lopez *et al.*, 2012].

In recent years, the root of the *Beta vulgaris* L. plant has attracted considerable attention as a functional health promoting food [Chhikara *et al.*, 2019]. It is rich in valuable active compounds such as: betalains [Gengatharan *et al.*, 2015; Khan & Giridhar 2015; Sawicki *et al.*, 2016, 2017], phenolics [Kujala *et al.*, 2002; Ninfali *et al.*, 2017; Preczenhak *et al.*, 2019; Ravichandran *et al.*, 2012], saponins [Mikołajczyk-Bator *et al.*, 2016], and fiber [Lundberg *et al.,* 2008]. Beet juice contains a high level of biologically available antioxidants and many trace elements such as potassium, magnesium, zinc, iron, calcium, phosphorus, sodium, as well as vitamins: niacin and biotin [Wootton-Beard & Ryan, 2011].

Red beetroots and their bioactive compounds can inhibit lipid peroxidation, increase resistance to low density lipoprotein oxidation, and exert a cancer-preventing effect [Lechner & Stoner, 2019; Ninfali *et al.*, 2017].

Betalains are present in the tuberous part of the plant, giving its red-purple coloration. They can be divided into two groups: betacyanins (red-violet) and betaxanthins (yellow-orange). Betanin is the most abundant betacyanin in red beetroot, isobetanin is the second major one. Betalain profile in thirteen red beetroot varieties was investigated by Sawicki *et al.* [2016], who identified thirty betalains, including 18 betacyanins and 12 betaxanthins. Betanin and isobetanin prevailed among the betacyanins, whereas vulgaxanthin I or miraxanthin II among the betaxanthins, depending on the variety.

The first mention of fermented beetroot in Poland appeared in the Herbarium of Syreniusz, and one of the first studies on the processes taking place during borscht making were conducted by Panek [1905]. There is little research on biologically active compounds in fermented beet products. Our previous studies addressed changes of red beet juice composition during spontaneous and controlled fermentation [Czyżowska *et al.,* 2006] and changes of fermented juice composition during 6 months of cold storage [Klewicka & Czyzowska, 2011]. Studies of Sawicki & Wiczkowski [2018] were concentrated on the effect of boiling and fermentation of red beetroot products on betalain profiles and antioxidant capacities. These authors investigated betalain profile and content in fermented shredded beetroots at 7th and 14th day of fermentation. They identified fifteen betacyanins and their derivatives, and also two betaxanthins. They also controlled juices of fermented red beetroots during fermentation and observed 11 betacyanins and their derivatives as well as 4 betaxanthins. Fermentation reduced the content of betalains by 61–88%, however this decline was lesser in unpeeled beetroots.

To the best of our knowledge, there is no literature addressing changes in fermented beetroot products during long-term cold storage. Therefore, the aim of our study was to investigate the effect of long-term cold storage on the content of biologically active compounds (betalains and phenolics) and microorganisms in fermented beetroots and fermented beetroot juice.

MATERIALS AND METHODS

Material and its preparation

The beetroots of Wodan variety originated from Agricultural Farm Michał Sznajder/ Gospodarstwo Rolne Michał Sznajder (Karnice, Poland; 51°28'57"N, 16°50'27"E). Beetroots were washed and peeled only from hard parts. Next, they were grated using a Solia G450 vegetable cutter (AlexanderSolia GmbH, Remscheid, Germany).

Fermentation and storage conditions

Grated beetroots were fermented with 7% salt addition in December 2016 and stored in 200-L barrels made of certified PP5 polypropylene. For a month, they were fermented at a temperature of about 15–18^{o}C, for another month at a temperature of about 10–13^{o}C, and then transported to a cold room to be stored at 5^{o}C.

To obtain juice, fermented grated beetroots were pressed on a hydraulic fruit press and bottled. All these processes were carried out at the Agricultural Farm Michał Sznajder/ Gospodarstwo Rolne Michał Sznajder.

Three samples from 3 barrels were examined each time, giving the total number of 9 samples per one analysis per one product. Juice samples were centrifuged, diluted if necessary, and filtered before HPLC/LC-MS analysis, while grated beet samples were extracted with water in a 1 to 9 ratio at room temperature for 30 min.

Determination of total phenolics content

Total phenolics content (TPC) was determined using the Folin-Ciocalteu (F-C) reagent [Waterhouse, 2001]. The exact conditions of analysis were given in Czyzowska *et al.* [2015]. The results were expressed as gallic acid equivalent and calculated based on the weight/volume of samples collected for analysis.

Spectrophotometric betalain quantification

Red and yellow pigment contents were analyzed using the Nilsson's spectrophotometric method [Nilsson, 1970]. Samples were mixed with a phosphate buffer (pH 6.5), to ensure the absorbance between 0.3 and 0.8 at 538 nm. Absorbance was measured at 476, 538, and 600 nm using a Cecil CE2041 spectrophotometer (Cecil Instruments Limited, Cambridge, UK). The results were expressed as betanin or vulgaxanthin equivalents for red and yellow pigments, respectively.

Betalain profile determination by HPLC-DAD

A Finnigan Surveyor liquid chromatograph equipped with an autosampler, a diode array detector (Finnigan Surveyor--PDA Plus), and ChromQuest 5.0 chromatography software (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used. Separation was performed on a Spherisorb ODS2 column (250 × 4.6 mm, particle size 5 μm, Waters, Milford, MA, USA) protected with a guard column of the same material. All samples were filtered through a 0.45 μm filter prior to chromatography. The HPLC method described by Czyżowska *et al*. [2006] was employed. Eluent A consisted of 0.2% TFA and 10% HCOOH (65:35, v/v), and eluent B was prepared by mixing 100% acetonitrile and 10% 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 470 and 538 nm for betaxanthins and betacyanins, respectively. Results were expressed as peak area (PA).

LC-MS^n analysis of bioactive compounds

The HPLC was coupled on-line with an MS LTQ Velos mass spectrometer (ThermoScientific, Waltham, MA, USA). Chromatographic separation was performed using a Hypersil Gold column (150 × 2.1mm, particle size 1.9 μm, ThermoScientific, Waltham, MA, USA). The mobile phase consisted of solvent A (1 mL formic acid in 1 L of deionized water) and solvent B (95% (v/v) acetonitrile). The analysis conditions were similar to those described by Nowak *et al*. [2016]. Elution began with 96% to 85% A for 8 min, from 85% to 82% A for 12 min, from 82% to 60% A for 40 min, from 60% to 50% A for 4 min, followed by washing and re-equilibration of the column. The injection volume was 10 μL. The flow rate was set to 220 μL/min. Electrospray ionization mass spectrometry was performed, in both the positive and negative ionization mode. Mass spectra were checked over the *m/z* range of 100–1000.

Determination of organic acids and ethanol contents

Organic acids were analyzed using a Finnigan Surveyor HPLC system (Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with an autosampler, a refractive index detector (Finnigan Surveyor-RI Plus), a diode array detector (Finnigan Surveyor-PDA Plus), and ChromQuest 5.0 chromatography software. Separation was performed on an Aminex HPX 87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) protected by a guard column. The analysis conditions were as given in Czyżowska *et al.* [2017]. The elution conditions were as follows: flow rate – 0.6 mL/min, oven temperature – 60°C, and solvent – 5 mM sulphuric acid.

Determination of microbial population count

Samples of the fermented grated beetroots and juice were prepared according to ISO 6887 [2010]. Total mesophilic count (TMC) was determined on a plate count agar (PCA) following incubation at 30°C for 96 h. Lactic acid bacteria (LAB) were quantified on De Man, Rogosa and Sharpe agar (MRS) following incubation at 30°C for 72 h under anaerobic conditions (Gas-Pack System, BBL, Becton–Dickinson, Franklin Lakes, NJ, USA). *Enterobacteriaceae* were determined on Violet Red Bile Dextrose agar (VRBD). Dichloran Rose Bengal Chloramphenicol agar (DRBC) was used for determination of yeast and mold counts. The lowest detection limit of these enumeration techniques was 10 CFU/g (CFU – colony forming units). Five samples of each product were analyzed. The results were expressed as CFU/g of beetroot or CFU/mL of juice.

Statistical analysis

All measurements were performed in nine independent replicates and the results are presented as mean values ± standard deviations (SD). The standard deviation was determined using STATISTICA 10 PL software (StatSoft, Krakow, Poland). The results were compared by one-way analysis of variance (ANOVA), whereas Tukey's test was carried out to test any significant differences among the mean values. Differences among mean values at 5% level ($P<0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Our previous investigations involved a six-month cold storage of fermented beetroot juices [Klewicka & Czyzowska, 2011]. There are no literature data about changes of fermented beetroot products after this time of storage. Therefore, we have decided to study changes of these products since the 7th month until the end of shelf-life.

Total phenolics content in fermented beetroot products

Total phenolics content in grated beetroot was approximately 900 mg/kg at the beginning of the experiment, while in juice it was approx. 800 mg/L at the same time (Table 1).

TABLE 1. Contents of total phenolics, and red and yellow pigments in fermented beetroot products during long-term cold storage.

Fermented product	Total phenolics content*	Red pigment**	Yellow pigment***
Grated beetroot (mg/kg)			
GB7M	920±120^a	116±11^a	14±1^b
GB8M	795±7^a	83±12^b	17±3^{ab}
GB9M	605±10^b	52±13^c	19±1^a
GB10M	570±5^c	38±2^c	13±2^b
Beetroot juice (mg/L)			
BJ7M	810±20^a	69±11^a	19±2^a
BJ8M	793±5^a	52±9^a	14±2^b
BJ9M	595±8^b	33±8^b	6±1^c
BJ10M	540±96^b	24±9^b	8±1^c

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in months. *Calculated as gallic acid equivalent. **Calculated as betanin equivalent. ***Calculated as vulgaxanthin equivalent. Data are expressed as mean±standard deviation, n=9. Different letters a-c for each product type indicate statistically significant differences ($p\leq0.05$).

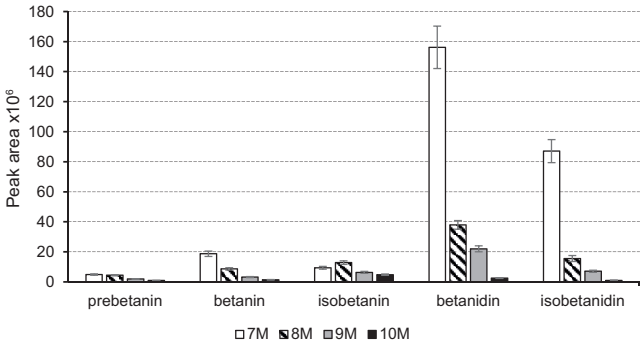

FIGURE 1. Quantitative composition of red betalains (betacyanins) in fermented grated beetroot during long-term storage at 5°C.

Content of compounds expressed as HPLC peak area. 7M-10M – time of storage in months.

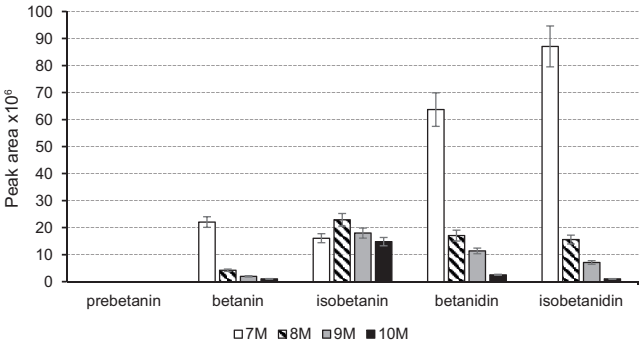

FIGURE 2. Quantitative composition of red betalains (betacyanins) in fermented beetroot juice during long-term storage at 5°C.

Content of compounds expressed as HPLC peak area. 7M-10M – time of storage in months.

The total phenolics content in the samples analyzed by Kavalcová *et al*. [2015] ranged from 820 to 1281 mg/kg in different varieties of red beetroot, and in the samples analyzed by Wootton-Beard & Ryan [2011] the content of phenolics was from about 620 to 1450 mg/kg, whereas their concentration in juices made of seven beet varieties ranged from 0.85 to 1.29 g/L [Wruss *et al.*, 2015]. As it can be seen, the concentration of this group of compounds in the investigated fermented grated beetroots and juices was comparable with the concentration of phenolics in fresh beets and in beet juices.

A downward trend was observed in phenolics content during storage (Table 1). The largest decrease was noticeable at the turn of the 8th and the 9th month of storage. At the end of the experiment, the content of phenolics was approx. 570 mg/kg in grated beetroot and 540 mg/L in beetroot juices. This could be due to the enzymatic oxidation of phenolic compounds in fragmented plant tissues and the activity of lactic acid bacteria enzymes, as well as oxygen access during the collection of subsequent batches of the products.

Betalain content

The concentration of betalains determined with the Nilson's spectrophotometric method [1970] was 116 mg/kg and 69 mg/L for red pigments and 14 mg/kg and 19 mg/L for yellow ones, for grated beetroot and juice, respectively. Study results indicate that red pigments predominated in the analyzed products. Their content in grated beetroots was about 8 times higher compared to the yellow ones (betaxanthins); while in the case of beetroot juice this difference was smaller (Table 1).

Both in the case of fermented beets and juice, about 3-fold decrease of red pigments content was observed during storage. Taking into account yellow pigments, their content did not change in grated beetroot, and their about 2-fold loss was observed in juices. These observations are consistent with literature data stating that betalains degrade during storage or processing which cause color changes [Esquivel, 2016].

As we stated before [Czyżowska *et al.*, 2006], for product color, not only the total content of the pigments is important but also their composition. The content of the main compounds (red betalains) found in fermented grated beetroots and fermented juice was also determined by HPLC. The results expressed as peak area (PA×10^6) are presented in Figures 1 and 2, respectively. Five main compounds from the betacyanin group were found in fermented grated beetroot: prebetanin, betanin, isobetanin, betanidin, and also isobetanidin. Prebetanin concentration in fermented juices was below the limit of quantitation.

Betanidin was shown to be the major compound among grated beetroot pigments at the beginning of the investigation, its content was above 50% of the total content of these pigments. The beetroot juice was predominated by isobetanidin (46%) and betanidin (34%). Their high concentrations can be effected by β-glucosidase activity of lactic acid bacteria (LAB) involved in the fermentation process. The activity of this enzyme is widespread among LAB typical of plant materials. β-Glucosidases release plant secondary metabolites from their β-*D*-glucosylated precursors [Michlmayr & Kneifel, 2014].

In most cases, the content of pigments decreased during storage, which could be influenced by many factors such as: matrix pH and water activity, enzymes activities, heat treatment, and exposure of product to oxygen and light during storage, as well as storage temperature [Belhadj Slimen *et al.*, 2017; Herbach *et al.*, 2006].

Minor increase of isobetanin content was observed between the 7th and the 8th storage month. Betacyanins, due to the C15 chiral center, exist in two epimeric forms, *e.g.* betanin and isobetanin. Epimerization can occur in an acidic medium and lead to an increased isobetanin concentration [Belhadj Slimen *et al.*, 2017; Herbach *et al.*, 2006].

Towards the end of the study, the proportion of pigments changed slightly, betanidin accounted for 45% and 30% of total betalains in grated beet and beet juice, respectively.

Compared to our previous research [Klewicka & Czyzowska, 2011] into the storage of fermented beet juice, the content of betanidin and isobetanidin is much higher, while the concentration of betanin and isobetanin is lower. This might be due to a longer storage time (up to 10 months), other fermentation conditions (addition of starter cultures in the case of previous studies), and scale of the research (laboratory and larger farm).

Considering our other research into the effect of supporting the fermentation of beet juice by the addition of a starter

TABLE 2. Tentative LC-MS^n identification of betalains in fermented products between the 7th and the 10th month of storage.

RT (min)	λ_{max} (nm)	$[M+H]^+$ *m/z*	MS^2 *m/z*	$[M-H]^-$ *m/z*	MS^2 *m/z*	Tentative identification	GB*	BJ*
2.28	263, 274, 460	273		271		portulaxanthin	+	+
3.36	475	459	295, 413, 251	457		unknown bidecarboxy-xanneobetanin isomer	+	+
3.62	284, 462	347		345		dopamine-bx (miraxanthin V)	+	+
4.32	280, 308	317		315	152, 162, 108	dopamine	+	+
4.70	280, 479	345		343	162, 180, 136	unknown decarboxy--betanidin isomer	+	+
5.06	483, 275	309		307		indicaxanthin	+	+
5.24	276, 479	391		389		dopaxanthin	+	-
5.78	536	505	356, 194			unknown decarboxy--dehydrobetanin isomer	+	+
5.93	537	551	389	549		betanin	+	+
6.05	535	713		711		2'-*O*-glucosyl-isobetanin	+	+
6.11	537	631		629		prebetanin	+	+
6.35		505	356, 194	503		unknown decarboxy--dehydrobetanin isomer	-	+
6.92	536	551		549		isobetanin	+	+
7.13	547	527				ni	-	+
7.23	542	389	343, 345, 150, 194, 258, 301, 178	387	299, 194, 343	betanidin	+	+
7.46		326		324		asparagine-betaxanthin (vulgaxanthin III)	+	-
7.63		311		309		valine-betaxanthin	-	+
8.35	542	389		387		isobetanidin	+	+
8.65	467	295	166, 120, 278	293	128, 275	unknown betanidin derivative	+	+
8.70	532	549		547	459, 297, 503, 415	neobetanin	+	+
9.07	280, 450	505				unknown betanidin derivative	+	+
9.80	280, 504	380		378	272, 306, 288, 254, 360, 179	ni	+	+
10.00	277, 527	231	214, 158, 188			ni	+	+
10.10	280, 482	325				vulgaxanthin IV	-	+
10.48	274, 456	461		459		2,17-bidecarboxy--neobetanin	+	+
10.35	277, 482	597				ni	+	-
10.58	279, 327, 482	323		321	128, 171, 303, 215	ni	+	+
12.25	465	331		329		tyramine-betaxanthin (miraxanthin III)	+	+
14.12	507	463		461		2,17-bidecarboxy--betanin/isobetanin	+	-
14.31	459	297		295		γ-aminobutyric acid--betaxanthin	+	+
14.52	532	727	551			6-*O*-feruloyl-betanin	+	-
21.32	534	637	593, 551, 389			isobetanidin 5-*O*-(6-*O*--malonyl)-β-glucoside	+	+

RT – retention time; GB – fermented grated beetroot, BJ – fermented beetroot juice, ni – not identified; * – presence of compounds at each time of storage (7M, 8M, 9M, 10M).

TABLE 3. Tentative LC-MS^n identification of other bioactive compounds in fermented products between the 7th and the 10th month of storage.

RT (min)	λ_{max} (nm)	$[M+H]^+$ *m/z*	MS^2 *m/z*	$[M-H]^-$ *m/z*	MS^2 *m/z*	Tentative identification	GB	BJ
2.18		329		327		betagarin	+	-
2.89	257, 470	259	163, 259	257		caffeic acid derivative	+	+
3.03	279, 320	181		179	135, 119, 92	caffeic acid	+	+
3.09	223, 280	155	137	153		protocatechuic acid	+	-
3.79	223, 276	139		137		*p*-hydroxybenzoic acid	+	-
4.32	280, 308	317		315	152, 162, 108	dopamine	+	+
4.77		166		164		ni	+	+
4.85		339		337		*p*-coumaroylquinic acid	+	+
5.86	364	321				2-*O*-(3,4-dihydroxy benzyl)-2,4,6- -trihydroxyphenylacetic acid	-	+
6.03				289	128, 271, 215	(epi)catechin	+	-
6.68	272, 310	293				ni	+	-
7.06		611		609		quercetin-glucoside	+	-
7.13	547	527				ni	-	+
7.81	271	345		343	192	theogallin	+	-
8.19	316	194		192	148, 174	ni	+	+
9.45	280, 491	571		569	371, 327, 389, 197	ni	+	+
9.80	280, 504	380		378	272, 306, 288, 254, 360, 179	ni	+	+
10.00	277, 527	231	214, 158, 188			ni	+	+
10.35	277, 482	597				ni	+	-
10.58	279, 327, 482	323		321	128, 171, 303, 215	ni	+	+
11.65	279			575	443, 425, 267	(epi)catechin-(epi) catechin (A type)	+	+
12.19	328	309		307		ni	+	+
12.85	225, 280	187	170, 158, 144			ni	+	-
13.06	280, 229			195	136, 151, 177	ni	+	+
17.03				301		quercetin	+	+
18.29	333			307	261, 97	ni	+	-
19.02				197	153, 180, 171, 136, 182, 93, 198	syringic acid	+	-

RT – retention time; GB – fermented grated beetroot, BJ – fermented beetroot juice, ni – not identified; * – presence of compounds at each time of storage (7M, 8M, 9M, 10M).

culture [Czyżowska *et al.*, 2006], it was noted that the addition of *Lactobacillus plantarum*, a plant-derived strain, caused enhanced synthesis of betanidin, compared to other bacterial strains. The influence of the variety on the proportions of the compounds tested was noted as well.

LC-MS identification of bioactive compounds in fermented beetroot products

To the best of our knowledge, there is no literature available regarding the betalain profile in fermented juices and grated beets during long-term cold storage. Available data relate to changes in this group of compounds during fermentation [Sawicki & Wiczkowski, 2018; Sawicki *et al.*, 2019].

The profile of bioactive compounds in fermented products (between 7 and 10 months of storage) was analyzed using the LC-MS^n technique. Compounds were identified by comparison of mass spectra, λ_{max} and retention times of available standards (phenolic acids and flavonols) or previously published data [Nemzer *et al*., 2011; Sawicki *et al.*, 2016; Sawicki & Wiczkowski, 2018; Slatnar *et al.*, 2015].

TABLE 4. The pH and the concentration of organic acids and ethanol in the fermented products.

Fermented product	pH	Lactic acid	Acetic acid	Propionic acid	Ethanol
Grated beetroot (g/kg)					
GB7M	3.71±0.32^{a}	6.03±1.04^{b}	4.77±1.02^{ab}	1.02±0.32^{b}	8.98±0.67^{a}
GB8M	3.56±0.13^{a}	6.63±0.20^{b}	5.54±1.30^{ab}	1.73±0.12^{a}	7.42±0.35^{b}
GB9M	3.32±0.25^{a}	8.68±0.92^{a}	3.54±1.03^{b}	1.04±0.94^{ab}	7.78±0.45^{b}
GB10M	3.61±0.08^{a}	6.40±0.15^{b}	6.30±0.65^{a}	1.59±0.36^{ab}	4.15±0.25^{c}
Beetroot juice (g/L)					
BJ7M	3.92±0.31^{a}	5.18±0.42^{a}	4.10±0.82^{a}	0.78±0.48^{b}	6.04±3.98^{ab}
BJ8M	3.83±0.25^{a}	5.03±0.36^{a}	4.28±1.08^{a}	0.56±0.14^{b}	3.85±1.05^{b}
BJ9M	3.81±0.48^{a}	5.01±0.76^{a}	4.90±0.62^{a}	2.26±1.02^{a}	9.06±3.06^{a}
BJ10M	4.12±0.37^{a}	4.76±0.64^{a}	3.86±0.58^{a}	0.32±0.10^{b}	3.28±0.62^{b}

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in month. Data are expressed as mean±standard deviation n=9. Different letters a-c for each product type indicate statistically significant differences ($p \leq 0.05$)

As expected, the majority of compounds belonged to the betalain family (Table 2). In addition to the basic betalains (prebetanin, betanin, isobetanin, betanidin, and isobetanidin) characterized by HPLC, additional 22 compounds were identified including, among others, neobetanin and its derivatives like 2,17-bidecarboxy-neobetanin and bidecarboxy-xanneobetanin isomer.

Studies of Wybraniec & Michałowski [2011] confirmed the highest enzymatic oxidating activity of neobetanin at pH 3, which is similar to the conditions prevailing in our products, and other studies of Starzak *et al.* [2016] have shown that one of the main products of enzymatic oxidation was 2,17-bidecarboxy-xanneobetanin.

Nine of these compounds were found in fermented roots and juices investigated by Sawicki *et al.* [2019] and fifteen in the research by Sawicki & Wiczkowski [2018].

Taking into account betaxanthins, 8 compounds from this group were found in fermented grated beetroots and juices. Four of them (miraxanthin V, indicaxanthin, miraxanthin III, and γ-aminobutyric acid-betaxanthin) were detected in both investigated products. One of these compounds – miraxanthin V – was found in fermented beetroot juices obtained by Sawicki & Wiczkowski [2018]. However, these authors identified three other betaxanthins not occurring in our juices. The newest studies of the aforementioned authors [Sawicki *et al.*, 2019] indicated the presence of two betaxanthins (vulgaxanthin I and miraxanthin V) in fermented beet roots. These authors did not identify any traces of these compounds in juices obtained during the fermentation process of beetroots. We did not find any traces of vulgaxanthin I in products studied, which is not consistent with our previous data concerning fermented juices [Czyżowska *et al.*, 2006] as well as data obtained by Sawicki *et al.* [2018]. But according to other literature data [Sawicki & Wiczkowski, 2018], this compound disappeared after the 5th day of fermentation of red beet juice. However, it was present in the fermented beets examined by the authors. The lack of this compound in our fermented products may be due to long storage or the variety used.

Considering phenolic compounds (Table 3), some acids were detected, including: *p*-hydroxybenzoic, protocatechuic, syringic, caffeic, *p*-coumaroylquinic, and galloylquinic (theogallin) acids. We did not find ferulic acid, that was detected in beet juices investigated by Wruss *et al.* [2015], but we identified its derivative with betanin – 6-*O*-feruloyl-betanin, a compound previously identified by Slatnar *et al*. [2015] in peel and petiole of red beet, by Sawicki *et al.* [2016] in 13 red beet varieties, by Sawicki *et al*. [2018] in fermented red beet juice, as well as by Sawicki & Wiczkowski [2018] and Sawicki *et al.* [2019] in fermented beetroot and juices. Flavonoids identified were: (epi)catechin, A type dimer of (epi)catechin-(epi) catechin, quercetin and its derivative quercetin-glucoside, and betagarin – a compound identified by Kujala *et al.* [2002].

Organic acids and ethanol

Table 4 shows the concentration of organic acids and ethanol in fermented grated beetroot and juice after 7–10-month storage. On the basis of chromatographic separation, the presence of three acids was revealed in the tested samples. They were products of lactic acid fermentation. Of the acids, lactic acid showed the highest concentrations in the tested samples, *i.e*. 6.03–8.68 g/kg in fermented grated beetroots, and 4.76–5.18 g/L in juices, and in most cases did not differ significantly ($p>0.05$) during storage time. Acetic and propionic acids occurred at levels of 3.54–6.30, 3.86–4.90 g/kg and 1.02–1.73 and 0.32––2.26 g/L, in fermented grated beetroots, and juices respectively. About 50% ethanol loss between the 7th and the 10th storage month was observed in both products tested.

There are no literature data concerning organic acids content in fermented beetroot products. But taking into account other fermented products, like olives, the major organic acids were lactic acid as the main biochemical product of fermentation followed by acetic acid, the presence of which could be attributed to homo- or hetero-fermentative metabolism of LAB strains due to nutrient limitation, salt concentration, as well as to yeast metabolism [Arroyo-López *et al*., 2012; Blana *et al*., 2014].

TABLE 5. Microbiological quality of fermented beetroot products during long-term cold storage.

Fermented product	TMC	LAB	*Enterobacteriaceae*	Yeast and molds
Grated beetroot (CFU/g)				
GB7M	$(1.8\pm0.5)\times10^{5b}$	$(4.5\pm1.3)\times10^{5a}$	$<10^{a}$	93 ± 35^{a}
GB8M	$(5.2\pm3.6)\times10^{4c}$	$(4.5\pm1.9)\times10^{3c}$	$<10^{a}$	$<10^{b}$
GB9M	$(8.0\pm2.0)\times10^{5a}$	$(4.6\pm1.2)\times10^{5a}$	$<10^{a}$	$<10^{b}$
GB10M	$(7.0\pm2.5)\times10^{3d}$	$(5.6\pm2.5)\times10^{4b}$	$<10^{a}$	$<10^{b}$
Beetroot juice (CFU/mL)				
BJ7M	$(3.6\pm0.7)\times10^{4c}$	$(2.4\pm0.8)\times10^{5b}$	$<10^{a}$	$(4.0\pm0.4)\times10^{3a}$
BJ8M	$(2.4\pm0.7)\times10^{3d}$	$(8.3\pm1.8)\times10^{5a}$	$<10^{a}$	$(1.4\pm0.7)\times10^{3b}$
BJ9M	$(1.5\pm0.1)\times10^{6a}$	$(1.4\pm0.5)\times10^{6a}$	$<10^{a}$	$(1.3\pm0.3)\times10^{3b}$
BJ10M	$(2.8\pm0.2)\times10^{5b}$	$(2.2\pm0.3)\times10^{5b}$	$<10^{a}$	$(1.2\pm0.5)\times10^{3b}$

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in months; TMC – total mesophilic count, LAB – lactic acid bacteria. Data are expressed as mean±standard deviation, n=9. Different letters a-d for each product type indicate statistically significant differences ($p\leq0.05$).

Microbiology

Results of microbiological analyses of the investigated products during storage are presented in Table 5. The total number of mesophilic bacteria in the tested samples reached 10^3–10^5 CFU/g in grated beetroots and 10^3–10^6 CFU/mL in juices. In most cases, there were no significant differences ($p>0.05$) between the number of mesophilic bacteria and the number of LAB. In some cases (especially in juices between the 7th and the 8th month of storage), the number of LAB was slightly higher, which may indicate better recovery of these bacteria in the MRS broth. The results indicate that lactic acid bacteria predominated among the bacterial microbiota in the fermented products. Larger numbers of LAB were observed in the fermented juices samples.

The count of yeast was lower in the fermented grated beetroots: at the beginning of investigated period at the level of almost 10^2, and at the end of observation lower than 10 CFU/g. The higher count of yeast was found in the juices (10^3 CFU/mL). *Enterobacteriaceae* were not detected in the fermented grated beetroots and beetroot juices throughout the storage time. These results indicate a high quality of the investigated products, which is ensured by the manufacturer, and that they are safe for the consumers in terms of their microbiological quality. In the case of fermented grated beetroots at the end of the storage period, the counts of mesophiles and LAB decreased in a statistically significant manner ($p\leq0.05$) as compared to their initial counts. In the case of juices, the numbers of all microorganisms were almost at the same levels during the storage period.

The storage survival of lactic acid bacteria, including potentially probiotic strains, depends on the environment and the type of strain used. Peñas *et al*. [2010] observed a steady increase in the number of lactic acid bacteria, including *Lb. plantarum* and *Lb. mesenteroides* strains, used for fermentation of cabbage juice, during 3-month storage at 4°C. Gardner *et al*. [2001], found a decrease in the number of bacteria from 10^9 CFU/mL after fermentation to 10^6 CFU/mL on the 90th day of storage of cabbage, beet, and carrot juices at 4°C, fermented with a mixture of *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains. According to these authors, the viability of bacterial cultures may result from the specificity of the strain used and the type of the food matrix (vegetables from which the juice was obtained). In turn, Yoon *et al.* [2005] studied the survival of LAB strains in fermented (72 h, temp. 30°C) beetroot juice, stored at 4°C for 4 weeks. The survivability of the strains was observed at levels ranging from 10^6 to 10^8 CFU/mL, except for *Lb. acidophilus* LA39. Finally, a research by Klewicka & Czyżowska [2011] regarding the storage of fermented beet juices with the addition of starter cultures showed the survival of LAB at 10^6 CFU/mL after 180 days of storage at 4°C.

CONCLUSIONS

This is the first report on biologically active compounds (betalains and phenolics) composition in fermented grated beetroots and beetroot juices during long-term cold storage.

The total phenolics content decreased during the process, and at the end reached 570 mg/kg in grated beetroot and 540 mg/L in juices. Betalains and phenolics profiles in these products were characterized and it was found that there were both quantitative and qualitative differences between them. Lactic acid bacteria predominated among the bacterial microbiota of the fermented products.

The tested products were found to be a rich source of biologically active compounds, and their health-promoting potential was enhanced by the presence of metabolically active lactic acid bacteria. During long-term storage, the content of their bioactive compounds decreased, however, remained at a high level.

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