The effects of fermentation by different species of lactic acid bacteria on betalains and polyphenol profile and *in vitro* bioactive potential of red beetroot juice

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Abstract

In the current study, the effects of fermentation by *Lactobacillus acidophilus*, *Levilactobacillus brevis* or *Lactiplantibacillus plantarum* (La/Lb/Lp, 1−2.5%) and incubation (30/37 °C, C1/C2) of red beetroot juice on the profile of betalains and polyphenols (UHPLC-DAD-MS), and antioxidant capacity using photochemiluminescence (PCL) and spectrophotometric assays (DPPH/ABTS) was investigated. Additionally, anti-glycaemic (anti-AGEs) and anti-cholinergic (anti-AChE) potential *in vitro* was analysed. Fermentation distinctly initiated isobetanin and neobetanin formation and enhanced flavonoid concentration, emphasising rutin, kaempferol and (+)-catechin. The fermented juices inhibited protein glycation in the BSA-GLU model and showed high DPPH and ABTS values. LP2.5% juice was the only one indicating anti-AChE potential.

Keywords: Antioxidant activity, Betalains, Lactic acid bacteria, Polyphenols, Pro-health potential

1. Introduction

Red beetroot (*Beta vulgaris* L.), and its products are enjoying increasing popularity among consumers in Eastern Europe due to their rich content of bioactive compounds such as polyphenols, carotenoids, glycosides, proteins and vitamins [1]. The standout feature of red beetroot is its water-soluble betalain colorants, with red-violet betacyanins (betanin, isobetanin) and yellow-orange betaxanthins (vulgaxantine I and II) being the primary subclasses [2]. Betanin, in particular, is a predominant compound in red beetroot (300−600 mg/kg) [3] and constitutes 75−95% of the total betalains, while betaxanthins make up the remaining 5−25% [4].

The beetroot phytochemicals, with emphasis on betalains, demonstrate a strong antioxidant capacity, and participate in the modulation of numerous biological effects both *in vitro* and *in vivo* [2,5,6]. Beetroot extracts were found to be potent inhibitors of acetylcholinesterase - a key enzyme responsible for Alzheimer's disease. According to *in silico* trials, betanin, myricetin, and folic acid were found to be the top three beetroot AChE inhibitors [6]. Moreover, it was found that extracts from green part of *B. vulgaris* inhibit α-amylase, α-glucosidase, and advanced glycation end-products' (AGEs) formation [7], positively influencing glycaemic parameters [5].

The use of lactic fermentation in food processing is an effective way to extend the products shelf life

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but also to increase their nutritional and sensory value [2]. The forced fermentation allows producers to oversee the concentration of macronutrients, bioactive ingredients and to obtain a product with reproducible quality. Lactic acid bacteria (LAB) are crucial not only for the fermented product quality, they also promote antioxidant capacity, and other health benefits in the obtained food [2,8].

Although the stability of biologically active colorants upon lactic fermentation process of red beetroot juice has been extensively studied in literature [9,10], there is limited data on the impact of the fermentation process on the antiglycaemic and anticholinergic activity of the beetroot juice. Therefore, this study was addressed to characterize the effects of lactic fermentation of beetroot (*B. vulgaris* L.) juice using *Lactobacillus acidophilus*, *Lactobacillus brevis* (current name: *Levilactobacillus brevis)* and *Lactobacillus plantarum* (current name: *Lactiplantibacillus plantarum)* strains not only in terms of the profile of betalains and polyphenols (UHPLC-DAD-MS), but also to evaluate the changes in the juice antioxidant capacity (PCL_{ACW}, PCL_{ACL}, DPPH, ABTS), anti-glycaemic (anti-AGEs) and anticholinergic (anti-AChE) activity. Correlations between bioactive compounds and various parameters were also explored.

2. Material and methods

2.1. Chemicals

All standards, reagents, and solvents used in this research were of HPLC grade. The following reagents: acetylcholinesterase (AChE) from electric eels (type V), galantamine hydrobromide, 5,5′[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (ATCI), glucose (GLU), methylglyoxal (MGO), bovine serum albumin (BSA), aminoguanidine hydrochloride, Folin-Ciocalteu's phenol reagent, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(pyridyl-s-triazine) (TPTZ) were obtained from Sigma Chemical Co. (Poznań, Poland). The remaining reagents (all of reagent-grade quality) were supplied by POCh (Gliwice, Poland). ACW (hydrophilic condition) and ACL (lipophilic condition) kits for the photochemiluminescence (PCL) assay were received from Analytik Jena AG (Jena, Germany). Analyzed phenols included protocatechuic acid, m-hydroxybenzoic acid, chlorogenic acid, salicylic acid, caffeic acid, syringic acid, sinapic acid, ferulic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, gallic acid, o-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, trans-cinnamic acid, vanillic acid, ellagic acid, vitexin, rutin, catechin, quercetin, apigenin, kaempferol, orientin, naringenin and myricetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of red beetroot juice

Fresh red beetroots Czerwona Kula variety (*B. vulgaris* L. *subsp. vulgaris*) were bought from a local market in Olsztyn, Poland. The roots were washed in distilled water and manually diced. To obtain the red beetroot juice a juice extractor was used (Waring Commercial Juice Extractor WJX50, China). The fresh juice (FJ) was transferred to the sterile containers (previously sterilized at 121 °C for 15 min) and pasteurized (80 °C for 15 min) to eliminate vegetative microflora.

2.3. Fermentation of red beetroot juice

Red beetroot juice was subjected to fermentation using *Lactiplantibacillus plantarum* ATCC 8014 (Lp), *Levilactobacillus brevis* Lbbr 12A (Lb) and *Lactobacillus acidophilus* ATCC 3543 (La) from the strain collection of the Department of Food Microbiology, Meat Technology and Chemistry, Faculty of Food Sciences of the University of Warmia and Mazury in Olsztyn (Poland). The juice (100 mL) was inoculated with 1%, 2% or 2.5% (v/v) (Lp/Lb/La, 1–2.5%) of an active culture of the strains grown in optimal media (MRS broth sticks). Fermentation was conducted for 24 h at a temperature of 37 °C for *L. acidophilus* or 30 °C for *L. plantarum* and *L. brevis*. Pasteurized juices with no inoculation treated under the same conditions was considered as the control (juice incubated in 30 °C (C1) and 37 °C (C2)). Three independent fermentation experiments were carried out. After fermentation, all samples were stored at −24 °C until further analysis.

2.4. Total phenolic content (TPC) analysis

The measurements of TPC were performed in microplates (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) according to the procedure described previously by Horszwald & Andlauer [11]. The results were calculated as milligram of gallic acid equivalent (GAE)/mL.

2.5. Total betacyanins (TBcC), total betaxanthins (TBxC) and total betalains (TBC) contents

Quantitative determination of betalains was performed using the spectrophotometric method

according to the test of Stintzing, Schieber and Carle [12]. For this purpose, the sample was dissolved in McIlvaine buffer (pH 6.5) to achieve an absorbance of $0.8 \leq A \leq 1.0$. Betacyanins (TBcC) were expressed as milligrams of betanin per mL of red beetroot juice; while betaxanthins (TBxC) as milligrams of vulgaxanthin I per mL. The TBC was expressed as the sum of betacyanins and betaxanthins.

2.6. Chromatographic analysis

2.6.1. Betalains analysis

The chromatographic analysis of betalains was performed according to the method described by Sawicki et al. [13] with some modifications. Betalain compounds were determined using a liquid chromatograph (Nexera XR, Shimadzu, Japan) coupled with a mass spectrometer (LCMS-2020, Shimadzu, Japan). The analysis was based on scanning in positive ionization mode. The separation of compounds was performed on a Gemini C18, 1.7 μm particle size, 100 × 1 mm column (Phenomenex, Torrance, CA, USA) at a temperature of 40 °C and a mobile phase flow rate of 0.35 mL/min. Elution was performed using a solvent gradient system consisting of solvent A (0.012% aqueous formic acid with 2 mM ammonium formate) and solvent B (95% aqueous acetonitrile with 2 mM ammonium formate and 0.012% formic acid). The following time gradient was used: 5% B (0–1 min), 5–95% B (1–2 min), 95% B (2–7 min), 95–5% B (7–8 min) and 5% B (8–10 min). The sample injection volume was 10 μL. Characterization of individual betalains were performed based on retention time, wavelength, and parent ions with the previously published data [13,14]. The MS and UV-vis data of detected red beetroot betalains are presented in Table 1. Quantity of betacyanins and betaxanthins was calculated from UHPLC-DAD-MD peak area against betanin and vulgaxanthin I, respectively, as the external standards. The calibration curve (the ranges of 0.01–0.80 mg/mL and 0.01–0.95 mg/mL, respectively) was linear with correlation coefficients of 0.998 and 0.999, respectively.

2.6.2. Polyphenols analysis

The chromatographic analysis of polyphenols was performed according to the methodology described by Sawicki et al. [15]. Polyphenols qualitative and quantitative were carried out using a UHPLC system (Nexera XR, Shimadzu, Japan) coupled with a diode area detector (DAD) and mass spectrometer (LCMS-2020, Shimadzu, Japan). Measurement parameters were as follows: eluent 0.01% formic acid in water with 2 mM ammonium formate (A) and 0.01% formic acid in 95% acetonitrile solution with 2 mM ammonium formate (B); flow rate 0.15 mL/min; scanning in negative ionization; column Column C18 BEH (1.8 μm particle size; 100 × 2.1 mm; Waters, Warsaw, Poland); oven temperature was 50 °C; sample injection volume 10 μL. An analysis was conducted in the selected ion monitoring mode (SIM). Analysed compounds were identified based on their qualitative ions, retention times and λ_{max} value with the previously published data [15,16]. The MS and UV-vis data of detected polyphenols are presented in Table 2. The quantity of polyphenols was calculated from the UHPLC-DAD-MS peak area against commercially available standards. The phenolic compound concentrations of the

Table 1. The MS and UV-vis data of betalains detected in red beetroot juices fermented by different species of lactic acid bacteria.

No	Compounds	R_t [min]	λ_{max} [nm]	$[MS]^+$ (m/z)
Betacyanins				
B1	betanin	2.05	537	551
B2	15-decarboxy-neobetanin	2.21	485	505
B3	15-decarboxy-betanin	2.23	507	507
B4	betanidin	2.26	537	389
B5	2,17-bidecarboxy-neobetanin	2.30	455	463
B6	17-decarboxy-betanin	2.68	507	507
B7	isobetanin	2.69	537	551
B8	2-decarboxy-neobetanin	2.70	485	505
B9	isobetanidin	2.71	539	389
B10	2-decarboxy-betanin	2.98	507	507
B11	neobetanin	4.01	470	549
Betaxanthins				
B12	vulgaxanthin I	1.77	474	340

Rt – retention time; λ_{max} [nm] – absorption maxima; $[MS]^+$ (m/z) – parent ion.

Table 2. The MS and UV-vis data of polyphenols detected in red beetroot juices fermented by different species of lactic acid bacteria.

No	Compounds	R_t [min]	λ_{max} [nm]	$[MS]^-$ (m/z)
Phenolic acids				
P1	gallic acid	2.71	272	169
P2	salicylic acid	5.15	298	137
P3	caffeic acid	5.54	323	179
P4	chlorogenic acid	5.66	298	353
P5	vanillic acid	6.01	260/292	167
P6	ferulic acid	6.26	322	193
P7	*p*-coumaric acid	6.52	309	163
P8	benzoic acid	6.68	228/272	121
P9	ellagic acid	6.78	265	301
Flavonoids				
P10	rutin	5.76	257/355	609
P11	kaempferol	5.83	345	285
P12	(+)-catechin	5.91	242	289
P13	myricetin	6.01	268	317
P14	apigenin	9.32	267/336	269
P15	naringenin	9.75	288	271

Rt – retention time; λ_{max} [nm] – absorption maxima; $[MS]^-$ (m/z) – parent ion.

solutions ranged from 0.01 to 150 μg/mL, with correlation coefficients of 0.997–0.999.

2.7. Antioxidant capacity assays

2.7.1. Photochemiluminescence (PCL) method

The PCL measurement was performed using the PHOTOCHEM apparatus (Analytik Jena, Germany) according to the measurement protocols provided by the manufacturer. The antioxidant activity was analysed using ACW (hydrophilic condition) and ACL (lipophilic condition) kits as previously described [9]. The antioxidant capacity was calculated using a comparison with the Trolox standard curve (0.5–3 nmol) and expressed as μmol of Trolox equivalent (μmol TE) per mL of sample.

2.7.2. DPPH assay

The DPPH scavenging ability against DPPH radicals was determined with a method described by Horszwald & Andlauer [11]. The measurement were performed at a microplate reader (FLUOstar Omega) with the wavelength established at 517 nm. Results were presented as μmol TE/mL of sample.

2.7.3. ABTS assay

The ABTS radical scavenging assay was carried out according to the previous described method by Horszwald & Andlauer [11]. The measurement were performed at a microplate reader (FLUOstar Omega) with the wavelength established at 734 nm. Results were presented as μmol TE/mL of sample.

2.8. In vitro antiglycaemic activity

2.8.1. Bovine serum albumin with glucose assay

Inhibition of AGEs formation in the bovine serum albumin with glucose (BSA-GLU) test was assessed following the method of Szawara-Nowak et al. [17]. A mixture (1 mL) of D-glucose (1.0 M), BSA (10 mg/mL) and sodium azide (0.1 mg/mL) in phosphate buffer (0.1 M, pH 7.4) was incubated (55 °C for 3 days) with or without 1 mL of the analyzed extract dissolved in phosphate buffer. The obtained material (300 μL) was placed into 96-well plates, and the formation of AGEs was determined based on the measurements of fluorescence at $\lambda = 330$ nm (excitation wavelength) and at $\lambda = 410$ nm (emission wavelength) (FLUOstar Omega). The positive control was aminoguanidine. The mathematical formula provided by Szawara-Nowak et al. [17] was used to calculate the percent inhibition of AGEs formation by the tested extracts. The IC_{50} values were calculated from linear regression analysis ($R^2 = 0.992–0.999$).

2.8.2. Bovine serum albumin with methylglyoxal assay

Inhibition of AGEs formation in the bovine serum albumin with methylglyoxal (BSA-MGO) test was assessed following the method of Szawara-Nowak et al. [17]. One mL of a mixture of BSA (1 mg/mL), MGO (5 mM) and sodium azide (0.1 mg/mL) in phosphate buffer (0.1 M, pH 7.4) was incubated (37 °C for 7 days) with or without 1 mL of the tested extracts dissolved in phosphate buffer. The obtained material (300 μL) was placed into 96-well plates, and the formation of AGEs was determined based on the measurements of fluorescence at $\lambda = 340$ nm (excitation wavelength) and at $\lambda = 420$ nm (emission wavelength) (FLUOstar Omega). The positive control was aminoguanidine.

The mathematical formula provided by Szawara-Nowak et al. [17] was used to calculate the percent inhibition of AGEs formation by the tested extracts. The IC_{50} values were calculated from linear regression analysis ($R^2 = 0.991–0.998$).

2.9. In vitro anticholinergic assay

Inhibition of AChE was assessed by a colorimetric method described by Eldeen, Elgorashi and Staden [18]. In the analysis the following buffers were used: *buffer A* (50 mM Tris–HCl, pH 8), *buffer B* (50 mM Tris–HCl, pH 8, containing 0.1% BSA) and *buffer C* (50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$). The reaction mixture consisted of 25 μL of 15 mM ATCI in water, 125 μL of 3 mM DTNB in *buffer C*, 50 μL of *buffer B*, 25 μL of extract in deionised water (0.1–10 mg/mL) or negative control (water) or positive control (galantamine). The mixture was prepared in 96-well plates (350 μL, PS, Porvair, Bioanalytic, Poland). The absorbance of the mixture was measured at 405 nm every 45 s (five times), and then 25 μL (0.2 U/mL) of AChE in *buffer B* was added to each well. Next, the absorbance was read eight times every 45 s (TECAN.Infinite M1000 PRO, TK BIOTECH, Warsaw, Poland).

The mathematical formula provided by Eldeen et al. [18] was used to calculate the percent inhibition of AChE activity by the tested extracts. The IC_{50} values were calculated from linear regression analysis ($R^2 = 0.999$).

2.10. Statistical analysis

The data are presented as mean values ± standard deviations of triplicate measurements (n = 3). The differences between samples were analyzed by a one-way ANOVA with NIR Fisher test ($p < 0.05$). Principal Component Analysis (PCA) was carried out

to investigate the impact of fermentation by different species of lactic acid bacteria on red beetroot juices. Hierarchical cluster analysis was performed on juices to determine their dissimilarity extent. The statistical analysis was performed using STATISTICA 13.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. The profile of betalains

The profile of betalains was analysed by HPLC-DAD-MS, and the obtained results were presented in Tables 1 and 3. A total of twelve compounds were identified, including eleven compounds belonging to the group of betacyanins and one compound from the group of betaxanthins (vulgaxanthin I) (Table 1). Betacyanins found in the tested juices were betanin, isobetanin, and their derivatives (betanidin and isobetanidin, respectively), decarboxylated derivatives (17-decarboxy-betanin, 2-decarboxy-betanin), dehydrogenated and decarboxylated derivatives (2,17-bidecarboxy-neobetanin, 2-decarboxy-neobetanin) as well as one dehydrogenated betanin derivative (neobetanin). The presence of above-mentioned compounds in red beetroot products has already been confirmed in literature [9]. However, in the current study we additionally identified 15-decarboxy-neobetanin and 15-decarboxy-betanin. Differences in the number of identified compounds may result from different experimental conditions used during the fermentation process, which in turn may affect the analysed betalains profile [10].

As shown, betanin dominated in all the tested juices, and its content ranged from 54% (fermented juices) to 71% (FJ) of the total sum of identified betacyanins (Table 3). The fermented juices showed slight changes in betanin content compared to FJ. In contrast, almost half of betanin content was lost after the juice incubation at 37 °C compared to FJ. Alike, Sawicki et al. [19] observed almost 50% decrease in betanin content in 7-day fermented juice. These authors related that phenomenon to the conversion of betanin to betanidin and isobetanidin. According to Czyżowska et al. [10], 2-fold reduction in betanin content noted for red beet juices inoculated with 10% of *L. brevis* and *L. plantarum* was due to decarboxylation and/or cleavage of betacyanins initiated by the fermentation process (30 °C/48 h).

Except for juice incubated at 37 °C (C2) other tested juices showed distinctly higher isobetanin concentration, and the most distinct, 2-fold increase in isobetanin content was noted for juices inoculated with 1%–2.5% of *L. acidophilus* and 2.5% of *L. brevis*. Evidently, isobetanin was the second main compound of the processed juices, ranging from 14.5% (C1) to 18.6% (La1%) of total sum of identified betacyanins. As above-mentioned, the fermentation process used in the current study effectively initiated the conversion of betanin to isobetanin. The increase in isobetanin was accompanied by the appearance of its aglycone form (isobetanidin) in all the fermented juices. However, the concentration of isobetanidin was distinctly low, and it ranged from 0.18% to 0.45% of the total sum of identified betacyanins. Among the tested juices, Lp2% juice showed the highest concentration of isobetanidin. The formation of isobetanidin and betanidin upon fermentation of red beet juices has already been proved in the literature [20].

Unlike isobetanidin, betanidin was present in FJ, C1 and C2 juices. The most significant 25% increase in betanidin content was found for Lb1% juice, whereas incubation of the juice at 37 °C resulted in about 20% reduction in betanidin content (Table 3).

All the analysed juices showed the presence of dehydrogenated form of betanin (neobetanin) (Table 3). The content of neobetanin range from 0.3% (for C2) to 5.9% (for La1%) of the total sum of identified betacyanins. The fermented juices inoculated with *L. acidophilus* showed about 3-fold higher neobetanin content then FJ. Alike, about 2-fold increase in neobetanin content was noted for C1, Lb1%, Lb2% and Lp1% juices. As reported previously, different decarboxylation products along with their corresponding neo-derivatives were identified in aqueous solutions of betacyanin subjected to heat treatment [21]. The authors demonstrated that, the predominant mechanism of betanin degradation upon heating was the hydrolytic cleavage leading to neobetanin formation. However, the results obtained in our study indicated that incubation of the juice at 37 °C led to almost complete loss in neobetanin concentration.

Among the analysed juices, La2% was characterized by the highest concentration of 15-decarboxy-neobetainin (Table 3). The concentration of this compound was higher by 27.5% and 42.5% than in FJ and C2, respectively. Considering the content of neobetanin and 15-decarboxy-neobetainin in the tested juices, we may conclude that the latter compound was more easily generated as a result of betanin degradation and derivatization under the experimental conditions than the former one.

As indicated, 2-decarboxy-neobetanin appeared in all the fermented juices ranging from 0.32% (for La1%) to 0.53% (for Lb2%) of the total sum of identified betacyanins (Table 3). Whereas, all the analysed juices showed the presence of 2,17-

Table 3. Total content and qualitative composition of betalains (mg/L) in red beetroot juices fermented by different species of lactic acid bacteria determined by UHPLC-DAD-MS.

No	BETALAINS COMPOUNDS	SAMPLES											
		FJ	C1	C2	La1%	La2%	La2.5%	Lb1%	Lb2%	Lb2.5%	Lp1%	Lp2%	Lp2.5%
BETACYANINS													
B1	betanin	10.10 ± 0.80^{ab}	9.24 ± 0.11^{b}	5.67 ± 0.05^{c}	10.02 ± 0.28^{ab}	10.60 ± 0.45^{a}	10.55 ± 0.45^{a}	9.62 ± 0.23^{ab}	10.13 ± 0.79^{ab}	10.32 ± 0.82^{ab}	10.55 ± 0.34^{a}	9.63 ± 0.29^{ab}	9.52 ± 0.35^{ab}
B2	isobetanin	1.65 ± 0.04^{e}	2.35 ± 0.06^{d}	1.46 ± 0.04^{e}	3.44 ± 0.10^{a}	3.36 ± 0.03^{a}	3.39 ± 0.09^{a}	2.81 ± 0.08^{c}	3.10 ± 0.01^{b}	3.35 ± 0.18^{a}	2.82 ± 0.09^{c}	2.85 ± 0.04^{c}	2.67 ± 0.02^{c}
B3	neobetanin	0.46 ± 0.01^{f}	0.95 ± 0.02^{d}	0.03 ± 0.00^{g}	1.09 ± 0.02^{a}	1.05 ± 0.02^{ab}	1.05 ± 0.03^{ab}	0.95 ± 0.02^{d}	0.94 ± 0.01^{d}	1.03 ± 0.04^{bc}	0.94 ± 0.02^{d}	0.99 ± 0.01^{cd}	0.75 ± 0.03^{e}
B4	15-decarboxy-neobetanin	0.87 ± 0.02^{e}	1.06 ± 0.04^{bc}	0.69 ± 0.02^{f}	0.96 ± 0.02^{de}	1.20 ± 0.04^{a}	1.09 ± 0.03^{bc}	1.04 ± 0.06^{cd}	1.07 ± 0.02^{bc}	0.96 ± 0.07^{de}	1.15 ± 0.04^{ab}	0.95 ± 0.03^{de}	0.94 ± 0.01^{e}
B5	2-decarboxy-neobetanin	nd	nd	nd	0.06 ± 0.00^{d}	0.07 ± 0.00^{c}	0.08 ± 0.00^{b}	0.06 ± 0.00^{d}	0.10 ± 0.01^{a}	0.09 ± 0.00^{b}	0.08 ± 0.00^{c}	0.07 ± 0.00^{c}	0.06 ± 0.00^{d}
B6	15-decarboxy-betanin	0.51 ± 0.03^{f}	0.75 ± 0.04^{de}	0.73 ± 0.06^{de}	0.93 ± 0.04^{bc}	0.65 ± 0.03^{e}	0.76 ± 0.05^{de}	0.84 ± 0.06^{cd}	1.14 ± 0.05^{a}	0.79 ± 0.06^{d}	0.75 ± 0.04^{de}	1.05 ± 0.05^{ab}	1.06 ± 0.04^{a}
B7	2-decarboxy-betanin	0.02 ± 0.00^{f}	0.73 ± 0.03^{d}	0.47 ± 0.03^{e}	0.94 ± 0.03^{bc}	1.15 ± 0.04^{a}	1.02 ± 0.07^{abc}	1.05 ± 0.05^{ab}	0.73 ± 0.03^{d}	1.10 ± 0.10^{a}	0.94 ± 0.03^{bc}	0.89 ± 0.05^{c}	0.64 ± 0.03^{d}
B8	17-decarboxy-betanin	0.07 ± 0.00^{g}	0.62 ± 0.03^{cd}	0.44 ± 0.03^{f}	0.47 ± 0.03^{ef}	0.76 ± 0.03^{ab}	0.74 ± 0.03^{ab}	0.73 ± 0.03^{ab}	0.81 ± 0.05^{a}	0.71 ± 0.05^{bc}	0.62 ± 0.00^{d}	0.56 ± 0.04^{de}	0.54 ± 0.04^{de}
B9	betanidin	0.36 ± 0.03^{cd}	0.38 ± 0.02^{bcd}	0.29 ± 0.02^{e}	0.35 ± 0.03^{d}	0.35 ± 0.02^{d}	0.40 ± 0.03^{abcd}	0.45 ± 0.02^{a}	0.41 ± 0.00^{abc}	0.37 ± 0.02^{bcd}	0.42 ± 0.01^{ab}	0.38 ± 0.01^{bcd}	0.37 ± 0.01^{cd}
B10	isobetanidin	nd	nd	nd	0.06 ± 0.00^{b}	0.06 ± 0.00^{bc}	0.06 ± 0.00^{bc}	0.05 ± 0.01^{c}	0.06 ± 0.00^{bc}	0.05 ± 0.00^{c}	0.04 ± 0.00^{de}	0.08 ± 0.00^{a}	0.03 ± 0.00^{e}
B11	2,17-bidecarboxy-neobetanin	0.08 ± 0.01^{e}	0.13 ± 0.01^{d}	0.15 ± 0.00^{cd}	0.17 ± 0.02^{bc}	0.17 ± 0.01^{bc}	0.19 ± 0.00^{b}	0.23 ± 0.01^{a}	0.23 ± 0.00^{a}	0.23 ± 0.02^{a}	0.16 ± 0.01^{bc}	0.15 ± 0.00^{cd}	0.22 ± 0.02^{a}
	sum of betacyanins	14.12 ± 0.84^{e}	16.22 ± 0.25^{d}	9.94 ± 0.05^{f}	18.50 ± 0.33^{abc}	19.43 ± 0.59^{a}	19.33 ± 0.73^{a}	17.83 ± 0.53^{abcd}	18.71 ± 0.93^{ab}	18.99 ± 1.13^{ab}	18.47 ± 0.49^{abc}	17.60 ± 0.46^{bcd}	16.81 ± 0.48^{cd}
BETAXANTHINS													
B12	vulgaxanthin I	8.30 ± 0.11^{a}	1.70 ± 0.08^{b}	1.22 ± 0.07^{c}	0.26 ± 0.02^{g}	0.26 ± 0.02^{g}	0.28 ± 0.01^{g}	0.56 ± 0.01^{e}	0.42 ± 0.03^{f}	0.47 ± 0.02^{ef}	0.75 ± 0.04^{d}	0.52 ± 0.00^{ef}	0.53 ± 0.03^{ef}
	sum of betalains	22.41 ± 0.73^{a}	17.92 ± 0.17^{cd}	11.16 ± 0.02^{e}	18.76 ± 0.35^{bcd}	19.69 ± 0.61^{b}	19.61 ± 0.71^{b}	18.39 ± 0.51^{bcd}	19.13 ± 0.90^{bc}	19.47 ± 1.11^{bc}	19.22 ± 0.52^{bc}	18.12 ± 0.46^{bcd}	17.34 ± 0.51^{d}

The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$) in the same row. FJ − fresh red beetroot juice; C1 and C2 − control samples (not fermented red beetroot juice incubated in 30 °C (C1) and 37 °C (C2); La, Lb and Lp − samples of red beetroot fermented by *L. acidophilus* (La), *L.* (Lb), and *L. plantarum* (Lp) at different concentrations (1%, 2%, 2.5%); nd − not detected.

bidecarboxy-neobetainin. Its concentration was from 0.56% (FJ) to 1.5% (C2) of the total sum of identified betacyanins. The highest, almost 3-fold increase in the content of 2,17-bidecarboxy-neobetainin was noted for juices fermented by *L. brevis*. It can be concluded that under the experimental conditions, betanin converted more effectively to 2,17-bidecarboxy-neobetainin and 15-decarboxy-neobetainin than to 2-decarboxy-neobetanin under the experimental conditions.

The presence of 15-decarboxy-betanin, 2-decarboxy-betanin and 17-decarboxy-betanin was also identified in the analysed samples. The detected mono-decarboxylated derivatives exhibited a significant increase post-processing, with Lb2%, Lp2%, and Lp2.5% showing the most notable (2-fold, on average) rise in 15-decarboxy-betanin concentration. This compound comprised from 3.3% (for La2%) to 7.3% (for C2) of the total identified betacyanins in the processed juices. Fermented juices, particularly La2%, La2.5%, Lb1%, and Lb2.5%, demonstrated over a 50-fold increase in 2-decarboxy-betanin. Its content ranged from 3.8% (for Lp2.5% juice) to 5.9% (for La2% juice) of the total sum of identified betacyanins. The concentration of 17-decarboxy-betanin also increased after processing, ranging from 2.5% (for La1% juice) to 4.3% (for Lb2% juice) of the total identified betacyanins.

The above-mentioned mono-decarboxylated derivatives were also found in LC-MS profile of aqueous solutions of betalain-rich extract subjected to acidification (acetic acid) and heating at 85 °C [22]. As found by these authors, the formation of decarboxylated derivatives was distinctly dependent on the concentration of betanin/isobetanin and acetic acid. In our study, it can therefore be hypothesized that the observed increase in the sum of identified betacyanins is not only influenced by the experimental condition of the fermentation process but also by the initial concentration of betanin, which is the main substrate for formation of these derivatives during the applied processing [21,22]. However, further investigation would be required to confirm this hypothesis.

As shown, the sum of all individual betalains identified in FJ (22.41 mg/L) significantly decreased after the treatment applied (Table 3), and the observed reduction was as follows: FJ > La2% = La2.5% > Lb2% = Lb2.5% = Lp1% > La1% = Lb1% > Lp2% > C1 > Lp2.5% > C2. Evidently, the drop in the sum of betalains noted for the processed samples was due to a distinct and significant reduction of vulgaxanthin I - the second main compound of the FJ. Irrespective of the treatment used, most of the vulgaxanthin I content was lost with emphasis on the fermented juices. It was also found that the fermentation of red beetroot juice resulted in significant decrease (21%) in betalains concentration [19]. The results presented in the above-cited reference support our observations that vulgaxanthin I is one of the main compounds of fresh red beet juice, being also a highly unstable one. Therefore, the changes in its content during processing, significantly determined the concentration of total sum of betalains in the analysed juices.

3.2. The profile of polyphenols

Quantitative and qualitative analysis of polyphenols in the tested red beetroot juices was carried out using HPLC-DAD-MS. The obtained results have been shown in Tables 2 and 4. Among the individual polyphenols found in FJ, six and four compounds from the group of phenolic acids and flavonoids were detected, respectively. In the group of phenolic acids, caffeic acid was the major compound, constituting 34.6% of the sum of individual phenolic acids (TPaI). The second major compound was ferulic acid (25% of TPaI) followed by gallic acid (24.5% of TPaI), salicylic acid (7.6% of TPaI), benzoic acid (6.6% of TPaI), and vanillic acid (1.4% of TPaI). Among the identified flavonoids, the predominant compound was myricetin, concentration of which was 46% of the sum of individual flavonoids (TFI). Naringenin was the second main flavonoid (39% of TFI) of the FJ. Whereas, rutin and apigenin constituted 12% and 3.2% of TFI.

A commercial beetroot juice analysed by Płatosz et al. [23] showed twelve phenolic acids, including caffeic acid and ferulic acid. However, their concentration noted in the above-cited reference was distinctly lower (0.185 μg/mL and 0.045 μg/mL, respectively) compared to our results. The polyphenol profile studied in juices obtained from seven Austrian beetroot varieties was found to be variety-specific [24]. The content of gallic acid found by these Authors in the studied material (27.7–30.2 μg/mL) is consistent with our data (Table 4). However, caffeic acid concentration (5.78 μg/mL) in FJ was even 7-times lower compared to the values noted in our study.

In opposite to our results, Płatosz et al. [23] detected eight flavonoids with orientin as a major compound in red beetroot juice. However, no myricetin and naringenin were detected in the above-cited study. The content of rutin, in turn, was distinctly lower (0.037 μg/mL) relative to our data. On the other hand, Ben Haj Koubaier et al. [25] found myricetin in juice from red beetroot

Table 4. Total content and qualitative composition of polyphenols (mg/L) in red beetroot juices fermented by different species of lactic acid bacteria determined by UHPLC-DAD-MS.

No	IDENTIFIED PHENOLICS	SAMPLES											
		FJ	C1	C2	La1%	La2%	La2.5%	Lb1%	Lb2%	Lb2.5%	Lp1%	Lp2%	Lp2.5%
PHENOLIC ACIDS													
P1	gallic acid	31.55 ± 0.01^{d}	nd	nd	32.60 ± 0.22^{b}	33.51 ± 0.05^{a}	33.28 ± 0.05^{a}	32.16 ± 0.08^{c}	31.70 ± 0.06^{d}	32.57 ± 0.03^{b}	32.46 ± 0.04^{b}	32.62 ± 0.00^{b}	32.18 ± 0.24
P2	salicylic acid	9.82 ± 0.41^{bcd}	7.64 ± 0.25^{h}	6.73 ± 0.03^{g}	10.22 ± 0.36^{ab}	9.89 ± 0.27^{bc}	10.09 ± 0.18^{ab}	9.21 ± 0.31^{de}	8.46 ± 0.20^{f}	9.19 ± 0.05^{e}	9.40 ± 0.13^{cde}	10.51 ± 0.11^{a}	9.99 ± 0.09^{abc}
P3	caffeic acid	44.52 ± 0.04	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
P4	chlorogenic acid	nd	nd	nd	2.26 ± 0.15^{d}	3.00 ± 0.23^{c}	3.00 ± 0.08^{c}	1.91 ± 0.08^{e}	3.64 ± 0.24^{b}	5.06 ± 0.07^{a}	3.39 ± 0.04^{b}	5.09 ± 0.08^{a}	4.91 ± 0.11^{a}
P5	vanillic acid	1.83 ± 0.36^{h}	nd	nd	10.94 ± 0.47^{a}	7.83 ± 0.05^{de}	8.59 ± 0.19^{bc}	6.09 ± 0.21^{g}	7.98 ± 0.21^{cde}	9.00 ± 0.12^{b}	8.27 ± 0.18^{cd}	7.43 ± 0.20^{ef}	6.78 ± 0.46^{f}
P6	ferulic acid	32.51 ± 0.23^{a}	25.87 ± 0.01^{b}	25.86 ± 0.01^{b}	25.57 ± 0.00^{cd}	25.43 ± 0.01^{d}	25.69 ± 0.05^{bc}	nd	nd	nd	nd	nd	nd
P7	*p*-coumaric acid	nd	nd	14.51 ± 0.01^{e}	15.10 ± 0.03^{a}	14.83 ± 0.08^{c}	15.02 ± 0.01^{b}	nd	nd	nd	14.67 ± 0.02^{d}	15.02 ± 0.04^{ab}	14.69 ± 0.03^{d}
P8	benzoic acid	8.53 ± 0.61^{a}	4.58 ± 0.13^{d}	nd	3.71 ± 0.36^{e}	5.58 ± 0.21^{c}	6.60 ± 0.06^{b}	nd	nd	nd	nd	6.56 ± 0.02^{b}	nd
P9	ellagic acid	nd	nd	nd	nd	nd	nd	nd	nd	12.64 ± 0.01	nd	nd	nd
	TPaI	128.74 ± 1.18^{a}	38.11 ± 0.11^{i}	47.10 ± 0.05^{h}	100.40 ± 0.09^{c}	100.08 ± 0.01^{c}	102.27 ± 0.23^{b}	49.37 ± 0.36^{g}	51.78 ± 0.12^{f}	68.46 ± 0.06^{e}	68.19 ± 0.32^{e}	77.24 ± 0.16^{d}	68.55 ± 0.16^{e}
FLAVONOIDS													
P10	rutin	7.64 ± 0.00^{f}	nd	nd	7.85 ± 0.01^{e}	7.89 ± 0.02^{d}	7.83 ± 0.00^{e}	8.04 ± 0.03^{b}	7.79 ± 0.01^{d}	8.03 ± 0.01^{b}	8.11 ± 0.01^{a}	8.11 ± 0.00^{a}	7.89 ± 0.01^{c}
P11	kaempferol	nd	nd	nd	30.48 ± 0.05^{a}	30.09 ± 0.03^{f}	29.90 ± 0.00^{g}	30.30 ± 0.02^{c}	30.41 ± 0.04^{b}	30.52 ± 0.01^{a}	30.23 ± 0.04^{de}	30.18 ± 0.00^{e}	30.28 ± 0.00^{cd}
P12	(+)-catechin	nd	nd	nd	12.33 ± 0.08^{bc}	12.59 ± 0.00^{b}	13.07 ± 0.06^{a}	12.02 ± 0.06^{cd}	12.07 ± 0.03^{cd}	12.18 ± 0.01^{cd}	nd	11.89 ± 0.08^{d}	11.31 ± 0.49^{e}
P13	myricetin	29.79 ± 0.05^{h}	30.43 ± 0.02^{ef}	28.96 ± 0.00^{i}	30.65 ± 0.07^{cd}	30.24 ± 0.04^{g}	31.00 ± 0.07^{b}	30.54 ± 0.06^{de}	30.34 ± 0.00^{fg}	30.54 ± 0.05^{de}	30.62 ± 0.06^{cd}	31.15 ± 0.01^{a}	30.73 ± 0.04^{c}
P14	apigenin	2.05 ± 0.00^{a}	1.98 ± 0.00^{b}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
P15	naringenin	25.21 ± 0.03	25.21 ± 0.00	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	TFI	64.68 ± 0.08^{f}	57.63 ± 0.02^{g}	28.96 ± 0.00^{h}	81.31 ± 0.21^{b}	80.81 ± 0.03^{c}	81.81 ± 0.01^{a}	80.90 ± 0.06^{bc}	80.71 ± 0.08^{cd}	81.28 ± 0.07^{b}	68.96 ± 0.00^{e}	81.34 ± 0.08^{b}	80.32 ± 0.53^{d}
	TPI	193.42 ± 1.26^{a}	95.74 ± 0.08^{j}	76.06 ± 0.05^{i}	181.71 ± 0.12^{c}	180.89 ± 0.02^{c}	184.08 ± 0.24^{b}	130.26 ± 0.43^{h}	132.49 ± 0.04^{g}	149.73 ± 0.01^{e}	137.15 ± 0.33^{f}	158.58 ± 0.24^{d}	148.87 ± 0.69^{e}

The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$) in the same row. FJ − fresh red beetroot juice; C1 and C2 − control samples (not fermented red beetroot juice incubated in 30 °C (C1) and 37 °C (C2]); La, Lb and Lp − samples of red beetroot fermented by *L. acidophilus* (La), *L. brevis* (Lb), and *L. plantarum* (Lp) at different concentrations (1%, 2%, 2.5%); nd − not detected; TPaI − Total phenolic acid index calculated by the sum of individual phenolic acids identified in the tested samples. TFI −Total flavonoids index calculated by the sum of individual phenolic acids identified in the tested samples. TPI − Total phenolic index calculated by the sum of individual phenolics identified in the tested samples.

cultivated in Tunisia. However, no rutin was detected in the analysed material. The different polyphenol profile presented in the above-cited literature compared to our research may result not only from differences in the plant variety, but also from a different degree of maturity, harvest period and/or storage conditions of red beetroots [23].

Caffeic acid was lost after the processing applied. Moreover, the loss in ferulic acid content was observed in the juices inoculated with *L. brevis* and *L. plantarum* (Table 4). All processed juices also indicated distinct drop in benzoic acid content compared to FJ, and its concentration ranged from about 4% of TPaI (for La1% juice) to 12% of TPaI (for C1 juice) (Table 4). In contrast, the process of fermentation led to significant increase in gallic acid content, i.e.: from 2% (for Lb2% and Lp2.5% juices) to 6% (for La2% and La2.5% juices). Gallic acid was found to be predominant compound in the fermented juices.

Fermentation increased salicylic acid concentration, and the most distinct increase (7%) was noted for Lp2% juice. Alike, the fermented juices showed significantly higher vanillic acid content compared to FJ and the most distinct, almost 5- and 6-fold increase was noted for Lb2.5% and La1% juices. The obtained results also showed that release of some polyphenols from the juice matrix occurred upon the processing applied with emphasis on the fermentation process. The appearance of chlorogenic acid was noted for all juices inoculated with LAB. The content of *p*-coumaric acid was found in the juice incubated at 37 °C, and in juices inoculated with *L. acidophilus* and *L. plantarum* (regardless of the inoculation volume). Its average content calculated for the tested juices was 31%, 15% and 21% of TPaI, respectively. Ellagic acid was only identified in the juice inoculated with 2.5% of *L. brevis*, and its concentration occurred at the level of 18% of TPaI.

Significant changes in the flavonoid profile were also observed after the processing applied (Table 4). Rutin was absent in the control juices, whereas, its concentration significantly increased in fermented juices. Alike, an increase in myricetin content was observed for majority of tested juices. Interstingli, myricetin was the only one flavonoid found in the C2 juice.

Apigenin and naringenin were not detected in the fermented juices, and in the juice treated at 37 °C. In contrast, fermentation induced the formation of kaempferol and (+)-catechin (Table 4). However, only, Lp1% juice did not show the presence of (+)-catechin. Kaempferol was present in the fermented juices at a similar level as myricetin. Its concentration ranged from 36% (for La2.5%) to 44% (for Lp1%) of TFI, whereas, (+)-catechin concentration was at the same level as an average (15% of TFI).

Despite a clear increase in flavonoids content in the fermented juices, a significant decrease in the sum of individual phenolics (TPI) was observed (Table 4). That phenomenon can be assigned to the loss in caffeic acid content and significant reduction in ferulic acid concentration. As evident both these phenolic acid were the major compounds of the FJ (23% and 17% of TPI). There is a scarce data regarding the changes in TPaI in fermented red beet juices. However, the available reports also indicated distinct reduction in phenolic acids upon fermentation. Płatosz et al. [23], noted almost 46% reduction in TPaI for spontaneously fermented red beetroots. Similar trends were observed in our study for the fermented juices, for which the reduction in TPaI was from 20% (La2.5% juice) to 61% (Lb1% juice). Compared to our data, Degirmencioglu et al. [26] obtained even lower TPaI values (59.91 μg/mL and 34.35 μg/mL) for red beet juices inoculated with *Saccharomyces cerevisiae* and *Sacchartomyces boulardii*, respectively.

As indicated from the obtained results (Table 4), the most pronounced drop in TPaI (70% and 63%), TFI (11% and 55%), and finally in TPI (50% and 61%) was noted for C1 and C2 juices, respectively. The observed phenomenon resulted not only from the temperature treatment used during the incubation of the tested juices, but could also result from the pre-treatment used, i.e. pasteurization of the juices. Considering all the above, we may conclude that the combination of both these thermal treatment used for the material preparation probably enhanced the degradation of polyphenols in the analysed juices. It was already reported that thermal processing of red beetroot had a negative impact on the product nutritional quality [27]. It should be emphasised, however, that it is difficult to compare our results with literature data as limited data can be found in terms of the detailed analysis of the profile of polyphenols in pasteurised and/or thermally incubated red beet juices.

3.3. Total phenolics, flavonoids, betacyanins, betaxanthins, and betalains content

The changes in the content of total phenolic (TPC) and total flavonoids (TFC) in processed juices were presented in Fig. 1A and B, respectively. As shown, both processes, i.e.: thermal incubation and lactic fermentation of juices, resulted in a significant reduction in TPC and TFC compared to FJ. Among all treated samples, the juices fermented with Lb1%

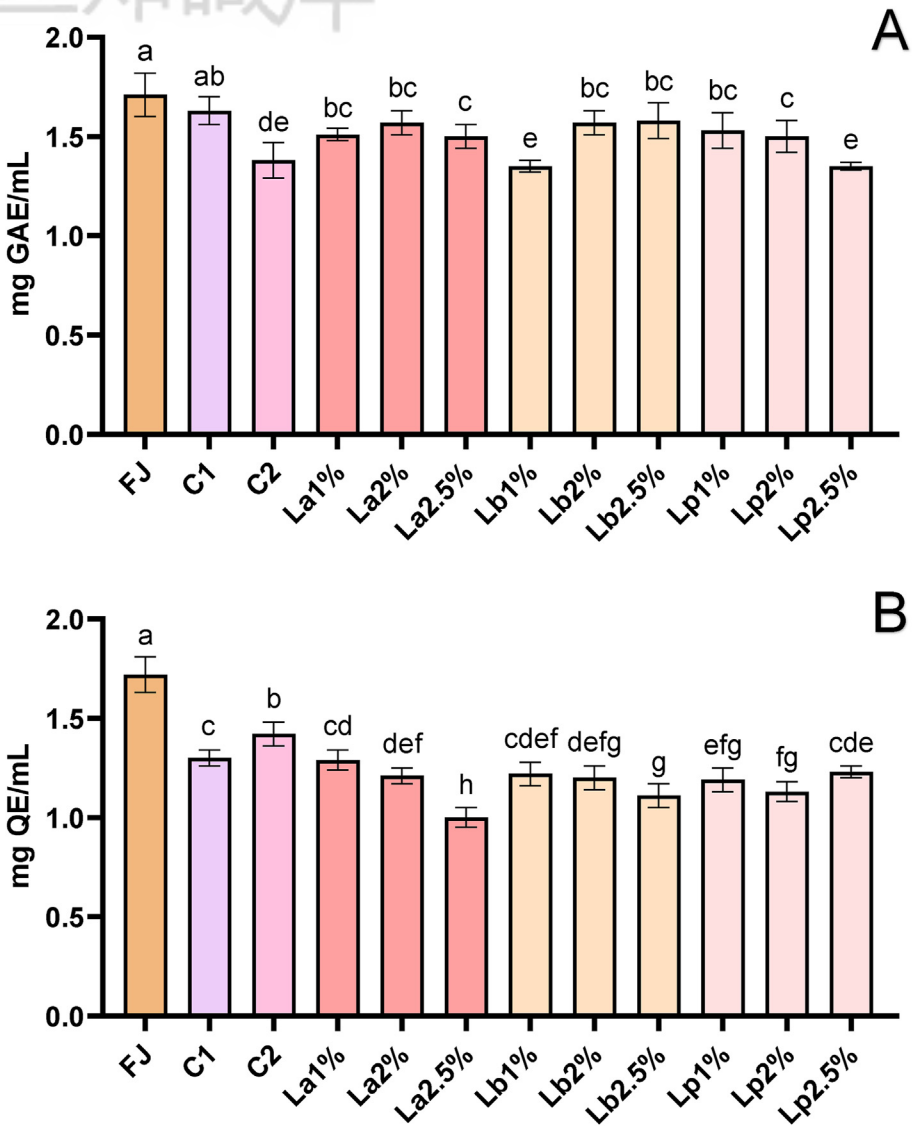

Fig. 1. Total phenolic (A) and total flavonoid (B) content in red beetroot juices fermented by different species of lactic acid bacteria determined by spectrophotometric methods. The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$). Abbreviations: FJ – FJ – fresh red beetroot juice; C1 and C2 – control samples (not fermented red beetroot juice incubated in 30 °C [C1] and 37 °C [C2]); La, Lb and Lp – samples of red beetroot fermented by L. acidophilus (La), L. brevis (Lb), and L plantarum (Lp) at different concentrations (1%, 2%, 2.5%).

and Lp2.5% showed the most distinct drop (nearly 21%) in TPC as reference to FJ. Whereas, La2.5% juice showed the most significant decrease (42%) in TFC.

Total content of betacyanins (TBcC) and yellow-orange pigments betaxanthins (TBxC) was analysed in the juices, as well. The total content of betalains (TBC) was calculated as the sum of TBcC and TBxC (Fig. 2). As shown, majority of treated juices showed minor reduction (2% on average) in TBcC values compared to FJ. Only the juice treated at 37 °C (C2) showed the most distinct drop (29%) in TBcC. In contrast, juices inoculated with 2% and 2.5% of *L. plantarum* had significantly higher (11%, on average) concentration of total betacyanins. Alike, C2 juice had the lowest TBxC as compared to the other samples. The resulting TBxC value was nearly 45% lower than the value found for FJ. However, the juice inoculated with 1%–2.5% of *L. acidophilus* and with 1%–2% of *L. brevis* showed similar degradation of TBxC which occurred at the level of 37%, on average. Among tested juices, the C1 juice manifested the lowest drop in TBxC (13%) as reference to FJ. Consequence of the changes in

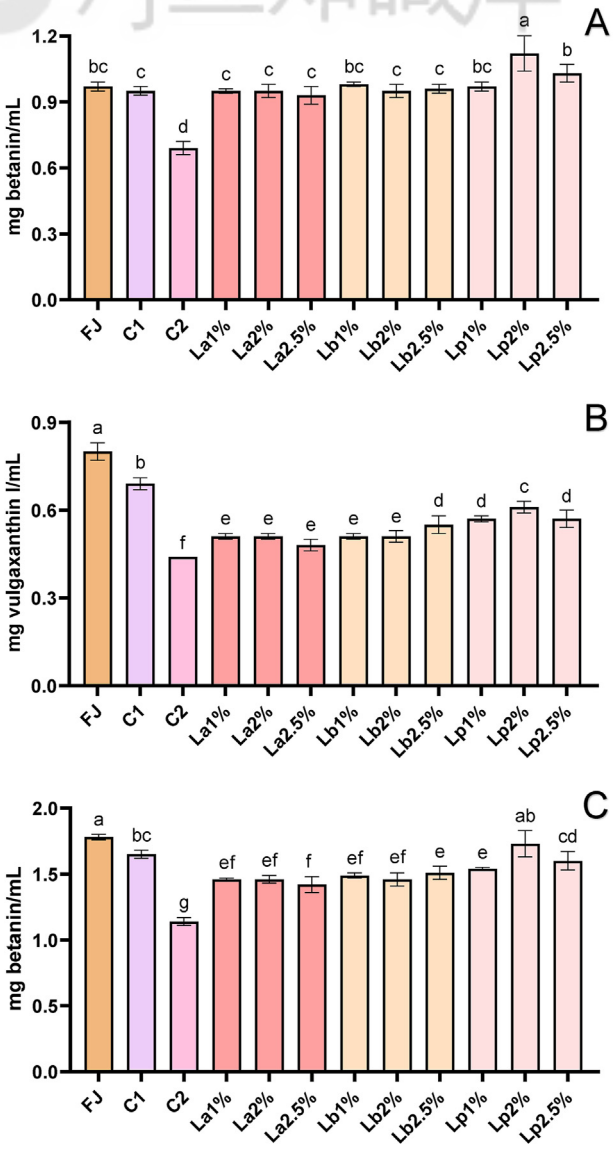

Fig. 2. Betacyanins (A), betaxanthins (B) and total betalains content (C) in red beetroot juices fermented by different species of lactic acid bacteria determined by spectrophotometric methods. The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$). Abbreviations: FJ – fresh red beetroot juice; C1 and C2 – control samples (not fermented red beetroot juice incubated in 30 °C [C1] and 37 °C [C2]); La, Lb and Lp – samples of red beetroot fermented by L. acidophilus (La), L. brevis (Lb), and L plantarum (Lp) at different concentrations (1%, 2%, 2.5%).

TBcC (Fig. 2A) and TBxC (Fig. 2B) are the TBC (Fig. 2C) values calculated for the tested samples. Among all the analysed samples, less visible changes in the obtained values were recorded for C1, Lp2% and Lp2.5% juices, where the applied treatment resulted in the reduction in TBC by 7%, 3% and 10%, respectively. Similar observation was made by Czyżowska et al. [10], who found a significant drop (8% and 13%) in the red pigment content in juice inoculated with *L. plantarum* and *L. brevis*, respectively. These authors also found that the treated juices had reduced yellow pigment content (i.e. 15% for Lp juice and 21% for Lb juice). These slight discrepancies in the presented values were probably due to different experimental conditions for fermentation, particularly the higher level (10%) of juice inoculation.

3.4. Antioxidant capacity

A combination of antioxidant tests, including spectrophotometric measurements (DPPH, ABTS) and photo-induced chemiluminescence (PCL), were used to assess changes in the antioxidant capacity (AC) of analysed juices (Table 5).

Scavenging activity against superoxide anion radical was analysed in the tested juices using hydrophilic and lipophilic condition (PCL_{ACW} and PCL_{ACL} respectively). The most significant decrease (32%) in PCL_{ACW} values was observed for La1% juice. Alike, juices inoculated with 2% of *L. acidophilus* and *L. brevis* showed distinct drop (21% and 25%, respectively) in AC. Only C1, Lb 1% and Lp1% juices had higher (by 7.26%, on average) PCL_{ACW} values compared to FJ. In contrast, PCL_{ACL} values noted for the analysed juices were significantly higher after the treatment applied. An average increase in PCL_{ACL} values observed for the studied juices was: C1 and C2 (41%) > Lb2.5% and Lp1%–Lp2% (21%) > La2% and La2.5% (16%) > Lp2.5% (10%) > Lb1% and Lb2% (5%). Whereas, only La1% juice showed 24% drop in PCL_{ACL} value. Similar phenomenon was observed in the work of Sawicki & Wiczkowski [9], who found a distinct increase (28%) in lipophilic AC for 1-day (spontaneously) fermented red beetroot juice. As reported, some aglycones of flavonoids (quercetin aglycone) and betacyanins (betanidin) demonstrated the antioxidant effect at varying polarities, and thus their activity can also be observed under lipophilic conditions [9]. Moreover, red beetroots are rich source of β-carotene, lycopene and lutein as well as vitamin E [28]. It can therefore be concluded that the analysed juices can be considered a complex matrix containing various antioxidants whose chemical nature, responsible for antioxidant activity, was modified as a result of the fermentation process used [20].

The C2 and La1% juices showed significant decrease in DPPH values, which occurred at average level of 8% (Table 5). In contrast, majority from the processed juices had higher DPPH values as compared to FJ. The observed increase in AC assayed with DPPH test was from about 2% (for La2% juice) to 28% (for LP1% juice). As evident, the

Table 5. The antioxidant capacity of red beetroot juices fermented by different species of lactic acid bacteria.

Samples	Antioxidant activity assays			
	PCL ACW	PCL ACL	DPPH	ABTS
	μmol TE/mL	μmol TE/mL	μmol TE/mL	μmol TE/mL
FJ	6.95 ± 0.08^{bcd}	7.93 ± 0.53^{d}	6.91 ± 0.48^{def}	2.87 ± 0.25^{e}
C1	7.92 ± 0.03^{a}	11.21 ± 0.87^{a}	8.01 ± 0.25^{b}	5.59 ± 0.19^{a}
C2	6.63 ± 0.20^{cd}	11.23 ± 0.05^{a}	6.33 ± 0.26^{f}	3.87 ± 0.29^{d}
La1%	4.75 ± 0.35^{h}	6.03 ± 0.00^{e}	6.41 ± 0.26^{ef}	5.02 ± 0.23^{cb}
La2%	5.49 ± 0.17^{fg}	9.08 ± 0.09^{bc}	7.02 ± 0.26^{cd}	5.57 ± 0.18^{a}
La2.5%	6.47 ± 0.00^{de}	9.31 ± 0.39^{bc}	7.58 ± 0.52^{bc}	5.11 ± 0.16^{bc}
Lb1%	7.14 ± 0.41^{bc}	8.33 ± 0.81^{cd}	6.91 ± 0.53^{def}	5.25 ± 0.14^{ab}
Lb2%	5.19 ± 0.06^{gh}	8.31 ± 0.34^{cd}	8.04 ± 0.40^{b}	4.96 ± 0.34^{bc}
Lb2.5%	5.90 ± 0.03^{f}	9.72 ± 0.55^{b}	7.25 ± 0.13^{cd}	4.84 ± 0.11^{c}
Lp1%	7.34 ± 0.12^{b}	9.65 ± 0.40^{b}	8.83 ± 0.59^{a}	4.78 ± 0.15^{c}
Lp2%	6.73 ± 0.34^{cd}	9.40 ± 0.17^{b}	7.48 ± 0.38^{bcd}	5.13 ± 0.25^{bc}
Lp2.5%	5.94 ± 0.51^{ef}	8.76 ± 0.54^{bc}	7.27 ± 0.32^{cd}	5.04 ± 0.11^{bc}

The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$) in the same column. PCL – photochemiluminescence method; ACW – hydrophilic condition; ACL – lipophilic condition; DPPH – DPPH radical scavenging assay; ABTS – ABTS radical scavenging assay; FJ – fresh juice; C1 and C2 – control samples (not fermented juice incubated in 30 °C (C1) and 37 °C (C2); La, Lb and Lp – samples of red beetroot fermented by *L. acidophilus* (La), *L. brevis* (Lb), and *L. plantarum* (Lp) at different concentrations (1%, 2%, 2.5%).

observed increase in DPPH values was strongly dependent on the type of bacteria used for inoculation, and the inoculation volume. In our previous study we did not observe antioxidant activity analysed with DPPH test for spontaneously fermented red beet juices [9]. On the other hand, Choińska et al. [20] observed more than 50% drop in DPPH values for juice inoculated with *L. brevis* and fermented for 7 days.

All the processed juices showed significantly higher ABTS values compared to the FJ (Table 5). The most distinct, almost 2-fold increase in AC, was noted for C1, for La2% and Lb1% juices. Similar trends were noted for La1%, La2.5%, Lp2% and Lp2.5% samples, for which the increase was 77%, on average. DPPH and ABTS assays are based by single electron transfer (SET)-reaction mechanisms, measuring the release of an electron and its conversion into an anion [29]. The ABTS assay's measurement of betalain's antiradical power was reported to be dependent on pH, with neutral and basic pH favouring deprotonation. Betaxanthins molecule is able to donate single electron from π-orbitals. However, the number of OH groups in betaxanthins enhances their activity in DPPH assay [30]. Flavonoids, particularly flavanols and flavonols, demonstrated the strongest AC in the DPPH tests, while naringenin lacks activity due to its low OH group count. Whereas, flavanols and hydroxybenzoic acids demonstrated low activity in ABTS tests [29]. Considering all the above, we may indicate that formation of kaempferol and (+)-catechin upon juices fermentation as well as the observed increase in the content of myricetin, gallic acid and vanillic acid were responsible for antiradical potential of juices measured with DPPH tests. Whereas, naringenin and kaempferol could be the most active antioxidants in ABTS assay. The presence of sugar residues slightly affected the antioxidant activity in the ABTS and DPPH tests [29], hence we can conclude that rutin (glycoside form of quercetin) found at the higher level in the fermented juices was responsible for their antiradical power in both tests used.

3.5. Anti-AChE and anti-AGEs activity

The inhibitory effect of the analysed juices on AChE activity and the formation of AGEs was examined by *in vitro* assays. The results obtained were expressed as IC_{50} values revealing the juice concentration (mg/mL) capable of inhibiting AChE and AGEs by 50% (Table 6).

Among all analysed juices, only Lp2.5% juice exhibited AChE inhibitory effect. There are only few data available in literature on the *in vitro* anti-AChE activity of red beetroot products. Rehman et al. [31] demonstrated strong anti-AChE activity of methanolic extracts of red beetroot, which was comparable to positive control (donepezil). The values (IC_{50}) obtained for the analysed samples were 3.73 and 3.69 μg/mL, respectively. Our data indicated similar anti-AChE effect of the galantamine (positive control) (Table 6), whereas the analysed potential of the Lp2.5% juice was distinctly lower as compared to the results noted in the above-cited reference. On the other hand, Desseva et al. [32] found no AChE-inhibitory effect for red beetroot juice. Notably, the authors did not explained the observed phenomenon.

Table 6. Inhibitory effects of red beetroot juices fermented by different species of lactic acid bacteria against acetylcholinesterase (AChE) activity and Advanced Glycation End-Products (AGEs) formation.

Samples	Inhibition (IC_{50}) [mg/mL]		
	anti-AChe	anti-AGEs	
		BSA-GLU	BSA-MGO
FJ	nd	2.64 ± 0.01^{c}	nd
C1	nd	2.35 ± 0.00^{b}	nd
C2	nd	1.94 ± 0.00^{a}	16.48 ± 0.96
La1%	nd	3.29 ± 0.04^{g}	nd
La2%	nd	3.10 ± 0.04^{efg}	nd
La2.5%	nd	2.91 ± 0.00^{de}	nd
Lb1%	nd	2.87 ± 0.14^{d}	nd
Lb2%	nd	3.00 ± 0.10^{def}	nd
Lb2.5%	nd	2.79 ± 0.05^{cd}	nd
Lp1%	nd	3.00 ± 0.18^{def}	nd
Lp2%	nd	2.64 ± 0.11^{c}	nd
Lp2.5%	0.73 ± 0.01	3.18 ± 0.03^{fg}	nd
Positive control			
Aminoguanidine (mg/mL)	–	0.109 ± 0.02	0.101 ± 0.02
Galantamine (μg/mL)	3.47 ± 0.01	–	–

The results are expressed as the mean ± SD. Different letters depict statistically significant differences ($p \leq 0.05$) in the same column. BSA – bovine serum albumin with glucose (GLU) or methylglyoxal (MGO) assay; FJ – fresh juice; C1 and C2 – control samples (not fermented juice incubated in 30 °C (C1) and 37 °C (C2); La, Lb and Lp – samples of red beetroot fermented by *L. acidophilus* (La), *L. brevis* (Lb), and *L. plantarum* (Lp) at different concentrations (1%, 2%, 2.5%); nd – not detected.

The lack of anti-AChE activity observed for majority juices may be attributed to the specificity (quality) of the examined material. The tested juices being complex matrix with antioxidants and other constituents such as sugars, organic acids, proteins, which can influence assay outcomes, as demonstrated in our previous study [33]. Removing high molecular weight compounds (HMW) through extraction and/or purification may distinctly enhance the results obtained from *in vitro* tests. On the other hand, it may be assumed that the inoculation of juice with 2.5% of *L. plantarum* was sufficient enough to modify not only the juice bioactivity through the formation and/or increase in the concentration of compounds from the group of betalains (Table 3) and polyphenols (Table 4) but mostly through the possible changes in the chemical nature of sugars. These LAB strains have an extensive set of metabolic pathways and enzymes capable of utilizing a wide range of HMW compounds, in particular sugars. Moreover, the juice was characterized by a high content of both (+)-catechin, rutin and chlorogenic acid-antioxidants with the ability to decrease AChE activity by binding with its active sites [34,35]. It was found that the number and position of OH groups in polyphenols enhance the inhibitory effects on AChE activity since hydroxyl groups promote the formation of hydrogen bonds with amino acids at the enzyme's active sites [36]. The highest anti-AGEs activity analysed in BSA-GLU model was observed for C1 and C2 juices (Table 6). Moreover, the juice exposed to 37 °C was the only one that showed the ability to inhibit the formation of AGEs in the BSA-MGO model. That finding is both interesting and surprising because the C1 and C2 juices were characterised by the lowest concentration and number of individual betalains (Table 3) and polyphenols (Table 4), content of which can be decisive for anti-AGEs activity of the plant foods [37]. On the other hand, only these juices had the highest antiradical potential measured by the PCL_{ACL} test (Table 5). As above-discussed, this phenomenon resulted probably from the potential role of lipophilic compounds present in the tested juices. Potentials candidates for this lipophilic compounds are carotenoids as suggested by Kaur et al. [38]. This authors found more than 2-fold increase in carotenoids content in red beetroot subjected to steam blanching and drying at 50 °C compared to the fresh sample (0.19 mg/100 g). It was also found that β-carotene, lycopene, and lutein showed strong inhibitory activity on AGEs formation in a heated model system at 65 °C [39]. However, to fully prove this hypothesis, further research is needed on the fate of lipophilic compounds during the process of fermentation and/or incubation of the red beet juice. The results obtained also demonstrated similar ability of fermented juices to inhibit the formation of AGEs in the BSA-GLU model (Table 6). The exposition of proteins to glucose initiated a nonenzymatic process of Maillard reaction, where reversible Schiff base adducts, and subsequently more stable Amadori rearrangement products are formed [40]. However, it was shown that antioxidants such as rutin and chlorogenic acid are capable of modifying amino or carbonyl groups in the Maillard reaction [41,42]. It can, therefore, be concluded that the fermented juices containing, among others, rutin and chlorogenic acid showed an inhibitory effect on albumin glycation in the BSA-GLU model. On the other hand, the content of antioxidants and their concentration in fermented juices was not sufficient enough to inhibit the formation of AGEs in the BSA-MGO model, in which more advanced glycation products were formed as a result of the activity of the main glycolytic precursor (MGO).

3.6. Principal Component Analysis

The principal component analysis (PCA) on 12 juices and 46 variables revealed that the first two components (PCs) accounted for 66.5% of the total variance (Fig. 3B). PC1 explaining 42.4% of the total

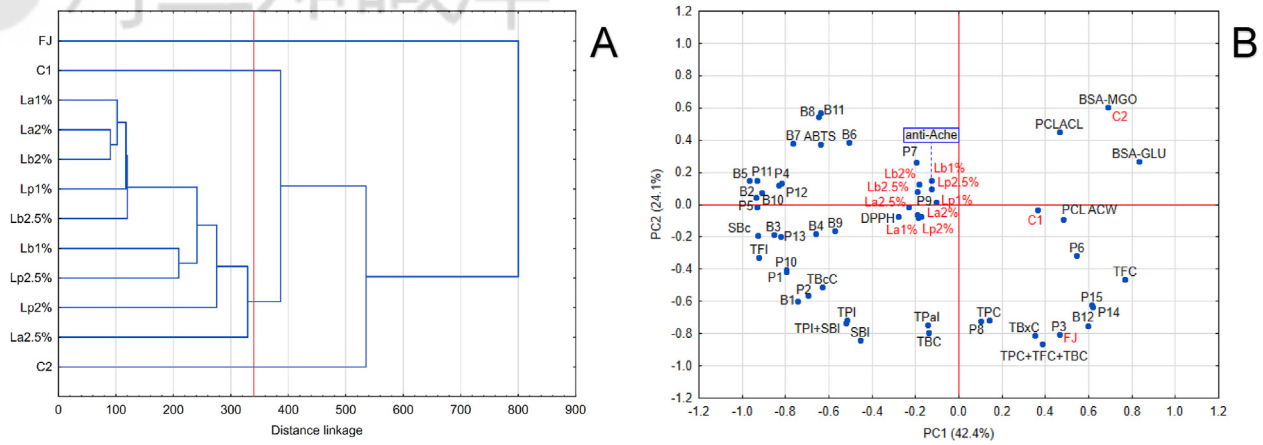

Fig. 3. The dissimilarity dendrogram among red beetroot juices (A). Principal component analysis biplot (B) of the total content and qualitative composition of betalains and polyphenols as well as antioxidant capacity and inhibitory effects against acetylcholinesterase activity and advanced glycation end-products formation variables for red beetroot juices against principal component 1 (PC1) and principal component 2 (PC2). Abbreviations: FJ – fresh red beetroot juice; C1 and C2 – control samples (not fermented red beetroot juice incubated in 30 °C [C1] and 37 °C [C2]); La, Lb and Lp – samples of red beetroot fermented by L. acidophilus (La), L. brevis (Lb), and L. plantarum (Lp) at different concentrations (1%, 2%, 2.5%).

variability and correlated positively with BSA-MGO, BSA-GLU, TFC and negatively with remaining parameters. PC2 explained 24.1% of the total variability among all analysed parameters and had weak positive variable influences and strong negative associations with SBI, TPaI, TPI, TPI + SBI, TBC, P8, TBxC, TPC, P3, and TPC + TFC + TBC.

PCA resulted in a distinct separation between the juices inoculated with LAB and control juices (C2, C1) as well as FJ. Fermented juices varied distinctly from C1, C2 and FJ, appeared to be unimodally distributed, and formed cluster considerably linked to the concentration of majority of identified betalains, polyphenols and SBc, TFI, ABTS, and TBcC parameters. The C2, C1 and FJ samples were separated from each other, and they were linked to different quality parameters. While, the distribution of the cluster formed by C2 juice was explained by PCL_{ACL}, BSA-MGO and BSA-GLU parameters, than C1 sample was mostly linked to BSA-GLU, PCL_{ACW}, and the concentration of ferulic acid. The distribution of FJ was connected with parameters such as: TBxC, TPC, TFC, TPC + TFC + TBC and the concentration of caffeic acid, naringenin, apigenin, benzoic acid, and vulgaxanthin I.

In addition to the PCA and better detection of relative similarity of difference between juice samples, cluster analysis (dendrogram) was applied (Fig. 3A). The general structure of the dendrogram showed the existence of four main clusters. The first cluster included FJ, while the second cluster contained C1. The fermented red beetroot juices created a third cluster, and four clusters included C2. The results indicate distinct differences in quality parameters between fermented and unfermented red beetroot juices. This highlights each juice's unique characteristics and associated bioactive compounds, offering valuable insights into their relationships.

4. Conclusions

To the best of our knowledge, this is the first work when the effect of forced fermentation using *L. acidophilus*, *L. brevis* or *L. plantarum* at different levels of inoculation (1–2.5%) of red beet juices on both the betalains and polyphenols profile as well as antioxidant capacity, anti-AGEs and anti-AChE potential *in vitro* has been studied. The obtained results showed that fermentation resulted in a significant increase in the sum of individual betacyanins. The fermentation process, emphasizing *L. acidophilus*, effectively initiated the conversion of betanin to isobetanin and the formation of neobetanin. All the fermented juices showed the content of isobetanidin and 2-decarboxy-neobetanin as well, as they were abundant in decarboxylated derivatives, dehydrogenated and decarboxylated derivatives, in particular 2-decarboxy-betanin and 2,17-bidecarboxy-neobetanin. Fermentation also led to the formation of kaempferol and (+)-catechin, as well as enhanced rutin and myricetin concentration. The juice incubated at 37 °C showed the strongest anti-AGE potential in both *in vitro* models, and the fermented juices effectively inhibited protein glycation in the BSA-GLU model. Only the juice inoculated with 2.5% *L. plantarum* indicated the anti-AChE potential *in vitro*. This study shed new light not only on the profile of betalains and polyphenols but also on the pro-health effect of red beet juices subjected to forced fermentation with La, Lb and Lp. The results

of this study may be helpful in the food industry in commercializing this technology for the development of novel fermented juices and also in promoting the nutritional strategy for the prevention of civilization diseases, particularly type 2 diabetes or Alzheimer's disease.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tomasz Sawicki: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Supervision, Writing – original draft, Writing – review & editing. Funding acquisition. **Monika Jabłońska**: Visualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Małgorzata Starowicz**: Methodology, Investigation, Formal analysis, Data curation. **Lucyna Kłębukowska**: Methodology, Investigation, Formal analysis, Data curation. **Wioletta Błaszczak**: Methodology, Visualization, Investigation, Formal analysis, Data curation, Supervision, Writing – original draft, Writing – review & editing.

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