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The *in vitro* digestates from Brussels sprouts processed with various hydrothermal treatments affect the intestinal epithelial cell differentiation, mitochondrial polarization and glutathione level

Joanna Doniec[1], Małgorzata Pierzchalska[2], Barbara Mickowska[1] and Maja Grabacka[2]

[1]Department of Plant Products Technology and Nutrition Hygiene, Faculty of Food Technology, University of Agriculture in Krakow, 122 Balicka St, 30-149 Cracow, Poland

[2]Department of Biotechnology and General Technology of Foods, Faculty of Food Technology, University of Agriculture in Krakow, 122 Balicka St, 30-149 Cracow, Poland

e-mail: maja.grabacka@urk.edu.pl

Brussels sprouts provide bioactive compounds with widely acknowledged health-promoting effects observed in various levels: single cells, organs and tissues or the whole organism. However, the choice of the appropriate hydrothermal processing is critical to sustain the nutritional values and cytoprotective activities, as Brussels sprouts are rarely eaten raw. The aim of this study was to evaluate the impact of various culinary methods (boiling, steaming and sous-vide in comparison to raw plant material) applied to Brussels sprouts on the chosen functions of liver and intestinal cell lines (HepG2 and Caco-2, respectively): the markers of enterocyte differentiation (alkaline phosphatase and sucrase-isomaltase activities and protein level), glutathione store management (total GSH concentration and Glu, Gly, Cys and Met amino acids analysis) and mitochondrial polarization (JC-1 staining analysis). The *in vitro* digestates from raw Brussels sprouts had stronger positive effect on the enterocyte marker enzymes in the Caco-2 cultures as compared to the digestates from the hydrothermally processed vegetables (boiled, steamed and sous-vide). The sous-vide method diminished the intracellular glutathione stores. Hydrothermal processing, particularly steaming and sous-vide had a negative impact on the mitochondrial abundance and polarization as compared to raw vegetables. Our results suggest that shorter processing is more beneficial to retain glutathione and mitochondrial polarization than longer hydrothermal treatment.

Key words: *in vitro* digestion, sucrase-isomaltase, alkaline phosphatase, boiling, steaming, sous-vide

Introduction

There is a common consensus of opinions among nutritionists and dieticians that vegetables and fruits should constitute at least half of every meal. *Brassica* vegetables are recommended and advised for consumption of minimum five portions a week, being, on the one hand, a low-calorie food with a high concentration of nutrients, minerals and fiber, and on the other hand, rich in components with health-promoting properties. The bioactivity of cruciferous vegetables is attributed to a high content of glucosinolates (GLS) and their metabolic products, such as isothiocyanates (ITCs) (Bogaards et al. 1994, Cartea and Velasco 2008, Verkerk et al. 2009, Johnson et al. 2015, Becker and Juvik 2016, Lafarga et al. 2018, Sanlier and Guler Saban 2018). The studies carried out with various tumor models underscore chemopreventive activities of GLS and ITCs, namely anti-inflammatory and immunomodulatory properties, inhibition of metastasis and angiogenesis, induction of apoptosis and cell cycle arrest (Gamet-Payrastre et al. 2000, Suppipat et al. 2012, Grabacka et al. 2014, Mitsiogianni et al. 2019), but their active participation in the maintenance of intracellular redox balance deserves special attention. GLS and ITCs from Brussels sprouts increase intracellular glutathione (GSH) level (Staack et al. 1998) and the expression of enzymes that protect from electrophiles and free radicals (Ye and Zhang 2001, Ernst et al. 2011), which helps the body to cope with oxidative stress. The molecular mechanism responsible for cytoprotection involves the activation of Nrf2 transcription factor, which binds to antioxidant response elements (ARE) in the promoters of genes encoding phase II detoxification enzymes: γ-glutamyl cysteine synthetase (GCL), a rate limiting enzyme of GSH production, heme oxygenase-1 and NAD(P)H:quinone oxidoreductase (Dinkova-Kostova and Kostov 2012). Sufficient GSH stores and efficient enzymatic machinery facilitate maintenance of mucosa physiological balance.

Most cruciferous vegetables, especially Brussels sprouts are rarely consumed in raw form and usually are processed, which directly affects their nutritional value, including amino acid provision. The most common hydrothermal processing methods differ in: temperature and duration, facilitation of release of nutrients from plant tissue, bioavailability of nutrients and GLS decomposition, which influence the ITCs content. Due to mostly negative effect

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of hydrothermal treatment on nutritional value of vegetables, new methods of processing are sought to enable obtaining a product with higher health and consumer quality. Sous-vide hydrothermal treatment (i.e. incubation of vacuum-sealed food portion in a controlled temperature) based on available research data is a promising processing method with regards to preserving health and nutritional value of *Brassica* vegetables. Limiting the loss of nutrients and bioactive ingredients during the sous-vide process is a result of vacuum packaging of the plant material prior to the proper thermal treatment. Direct contact of food with the water medium is limited, thus leaching of compounds is smaller. In addition, the negative impact of heat treatment is also minimized by using lower temperature and longer processing time, than in the case of conventional cooking, which further limits decomposition of nutrients, vitamins and phytochemical antioxidants. In addition, temperature is precisely controlled (Lafarga et al. 2018, Sanlier and Guler Saban 2018, Doniec et al. 2022a, Doniec et al. 2022b).

The meal composition and food processing method have a great impact on the development of physiological functions of intestinal epithelial cells, as well as the gut mucosa homeostasis. The expression and activity of enterocyte brush border enzymes, such as alkaline phosphatase and sucrase-isomaltase, determine digestive and absorptive functions of the intestinal epithelium. The efficient detoxification of oxidative stressors requires sufficient glutathione stores. Reduction of oxidative stress correlates with proper metabolic activity of mitochondria. Roughly 90% of intracellular superoxide anion production originates from incomplete reduction of molecular oxygen during the electron transfer through the mitochondrial respiratory chain (so called 'proton leak') (Indo et al. 2015), especially in metabolically active tissues, such as gut epithelia. Therefore, the maintenance of a proper balance between active respiration and removal of reactive oxygen species is crucial to support epithelial health and barrier functions. Dietary bioactive compounds from cruciferous vegetables are good candidates positively contribute to these two processes.

The aim of this study was to evaluate the impact of various hydrothermal processing methods applied to Brussels sprouts on the markers of enterocyte differentiation, as well as GSH store management and mitochondrial polarization in enterocyte- and hepatocyte-like cell cultures. We applied *in vitro* digestion to mimic the situation in the gut after consumption of Brussels sprouts prepared in different ways and to our best knowledge, the obtained results present novelty in respect to the influence of the digestates on the cell condition and functions.

Materials and methods

Plant material preparation

The applied hydrothermal treatments of plant material have been described in detail in previous works (Doniec et al. 2022a, Doniec et al. 2022b). Briefly, fresh Brussels sprouts (*Brassica oleracea* var. *gemmifera*) were purchased in a local supermarket (Cracow, Poland). Plant material was cleaned and split into four sub-sample groups corresponding to different types of hydrothermal treatments. First sub-sample was left raw, next ones were subjected to: steaming (100 °C; 7 min) sous-vide (90 °C at constant temperature control; 50 min) and traditional boiling in water (98 ± 1 °C; 15 min; plant material to water ratio was 1:3, w:v).

In vitro digestion of Brussels sprouts

In vitro digestion was conducted according to Zyla et al. (1995) with modification by Starzyńska-Janiszewska et al. (2016). Briefly, homogenized plant material (5 g) was placed in flask, then distilled water was added to a final volume of 10 ml and mixed afterwards. Then, the solution pH was adjusted to 2.0 with HCl (0.5 M) prior to adding 0.2 ml of pepsin solution (Sigma-Aldrich #P6887, Saint Louis, MO, USA; concentration 6 mg ml^{-1}; activity 3850 U mg^{-1}; dissolved in 0.1 M HCl) and placed in 37 °C shaking water bath (GFL 1092, 40 rpm min^{-1}) for 2 h, to simulate a gastric phase of digestion. Next, pH was adjusted to 7.0 with 1 M $NaHCO_3$ prior to adding 0.5 ml of pancreatin (#P7545, Sigma Aldrich, 90 mg ml^{-1}) and bile salt mixture (#B8631, Sigma Aldrich, 9 mg ml^{-1}), dissolved in 0.1 M $NaHCO_3$. The digestate was placed in 37 °C shaking water bath for 2 h, as previously, to simulate a small intestine phase of digestion. Finally, the samples were centrifuged at 1500 rpm for 15 min (MPW 352R Centrifuge, MPW Med. Instruments, Poland). Supernatants were freeze-dried and used for further analyses. For amino acid analysis the digested samples were dialyzed, as described in details elsewhere (Doniec et al. 2022a).

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The estimation of physiological amounts of *in vitro* digestates

The prepared digestates were applied in the cell culture experiments at physiological concentrations which were calculated based on Hidalgo et al. (2018). With regard to the latest recommendations of the Polish Institute of Food and Nutrition, half the volume of each meal should comprise vegetables and fruits, a single serving of Brussels sprouts was set as 100 grams. Thus, referring this value to the conditions and volumes used in *in vitro* digestion and the estimation of the intestine surface as 200 m^2, the physiological concentrations of Brussels sprout digestates were calculated as volumes (µl) per cm^2 of cell culture area, respectively: raw Brussels sprouts 0.317 µl cm^{-2}, boiled 0.325 µl cm^{-2}, steamed 0.29 µl cm^{-2} and processed by sous-vide 0.312 µl cm^{-2}. Additionally, a digestive enzyme and bile salts mix was used as a control sample 0.083 µl cm^{-2} (a solution of digestive enzymes in concentrations used during *in vitro* digestion).

Cell culture

Caco-2 human adenocarcinoma cells (ECACC 86010202, purchased from Sigma-Aldrich, USA, batch #08B011) were cultured using Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g l^{-1} D-glucose concentration and GlutaMAX addition (Gibco; ThermoFisher; USA) supplemented with 10% (v/v) fetal bovine serum (EurX, Poland) and antibiotic –antimycotic mixture (streptomycin 100 mg ml^{-2}, penicillin 100 U ml^{-1} and amphotericin B 250 ng ml^{-1}; Biowest, USA) in standard conditions (37 °C, 5% CO_2, 98% humidity). For the differentiation experiments, Caco-2 cells were seeded into upper compartments of 12-well or 6-well 0.4 µm PET Transwell inserts (50 000 and 460 000 cells, respectively). Both upper and lower compartments were filled with the cell culture medium; which was changed every second day. After the monolayers reached confluence, the differentiation progress was assessed by measuring of transepithelial electric resistance (TEER) using EVOM electrode (World Precision Instruments, Sarasota, FL, USA), on the indicated time points. HepG2 human hepatocellular carcinoma cells (ATCC HB-8065, batch #70039681) were cultured in the same conditions as Caco-2 cells, except for the DMEM medium with low glucose concentration (1.0 g l^{-1}, Gibco; Thermo Fisher; USA) was used.

Cytotoxicity assay

In order to determine the effect of physiological concentrations of *in vitro* digestates on the viability of Caco-2 and HepG2 cells, the MTS cytotoxicity test was performed (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega; Madison; USA), which is based on the assessment of metabolic activity of viable cells. Cells were seeded into 96-well plate (5000 cells per well) and left for 24 h to attach. Next, the physiological concentrations of digestates were added for 48 h and the assay was performed according to the manufacturer's instruction. Results are expressed as the percentage of nontreated cells.

Intestinal alkaline phosphatase (ALPI) activity

The determination of alkaline phosphatase activity was based on Ferruzza et al. (2012) with minor adjustments. In short, Caco-2 cells suspended in DMEM cell culture medium, placed in the upper 12-well Transwell chambers and left overnight for attachment. Next, cells were differentiated in the presence of digestates added in physiological concentrations to the upper compartments at each change of the medium, whereas the regular DMEM medium was added to the lower compartments. In both compartments the media were changed three times a week. Alkaline phosphatase (ALPI) activity was measured on 11th day after the culture had reached confluence. On the day of the assay, medium was drained from both upper and lower compartments, the cells were washed with Mg^{2+}/Ca^{2+} PBS. The PBS was removed and the reaction was started by adding 600 µl of 11.4 mM p-NPP substrate in 0.1 M bicarbonate buffer, pH=10 and carried out in the cell culture incubator (37 °C, 5% CO_2, 98% humidity). In several consecutive time points the reaction was stopped by transferring 80 µl of the reaction mixture to a 96-well plate, containing 20 µl of 0.5 M NaOH. In the meantime, a blank replicate (p-NPP substrate alone with 0.5 M NaOH) and a standard curve (0-200 µM of p-NP) were also performed. The absorbance was recorded at 400 nm. The ALPI activity was calculated as an initial rate (p-NP µmol $[l*min]^{-1}$).

Sucrase-isomaltase activity (SI)

The determination of brush border sucrase-isomaltase (SI) activity was based on (Messer and Dahlqvist 1966) with minor adjustments. Briefly, Caco-2 cells suspended in DMEM cell culture medium, placed in the upper 6-well Transwell chambers and left overnight for attachment. Next, cells were differentiated in the presence of digestates in physiological concentrations in the same pattern as for ALPI assay. The integrity of the cell monolayer during differentiation was monitored by TEER measurements. The SI assay was performed on the 2nd, 6th, 9th and 11th

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day after the culture reached confluence. On the day of the assay, the cells were washed twice with Mg^{2+} and Ca^{2+} containing PBS. PBS remained in the lower compartments and in the upper compartments PBS was successively replaced by 1 ml of 28 mM sucrose in Tyrode buffer (pH=6) at equal time intervals. Subsequently, cells were incubated with sucrose solution for 90 min in 37 °C, 5% CO_2, 98% humidity. In parallel, each of the conditions also had a control well to which Tyrode's buffer without sucrose was added. After the incubation, reaction mixture from above the cells was collected, centrifuged and the 500 µl aliquot was taken for the assessment of glucose concentration and mixed with 1.5 ml glucose oxidase – peroxidase – orto-dianisidine mixture (1 µg ml^{-1} glucose oxidase #G6125, Sigma-Aldrich, USA; 100 µl 1% orto-dianisidine ethanol solution, 100 µl horseradish peroxidase #P8250-25KU, Sigma-Aldrich USA, 1 mg ml^{-1} solution in Tyrode buffer). The GOX reaction mixtures were incubated for 60 min at 30 °C. The reaction was stopped by adding 2.5 ml of 2.5 M H_2SO_4. The absorbance was measured at 530 nm. The rate of glucose release from sucrose was calculated using the standard curve (nmol glucose min^{-1}).

Glutathione (GSH) determination

The total GSH concentration in Caco-2 and HepG2 cells was assessed Glutathione Colorimetric Detection Kit (#EIAGSHC; Invitrogen; Thermo Fisher; USA), according to the instruction. Briefly, the cells were lysed in assay buffer provided with the kit, supplemented with Triton X-100 to final concentration of 0.1% (v/v), the aliquots were deproteinized with 5% sulfosalicylic acid, vortexed, sonicated on ice for 30 s and then centrifuged at 14 000g, 10 min, 4 °C. The supernatants were mixed with NADPH, glutathione reductase and colorimetric reagent, incubated for 20 min and absorbance at 405 nm was recorded using a microplate reader. At the same time, the protein concentration in the cell lysates was determined using BCA protein assay (Quanti-Pro BCA Protein Assay Kit, Sigma Aldrich, USA, #QPBCA). The concentration of total GSH in the samples was normalized to the protein concentration in the lysates and expressed as pmol GSH $µg^{-1}$ protein.

Immunoblotting

Total protein extracts were prepared from differentiated Caco-2 cells using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Inc., Danvers, MA, USA #9803) supplemented with phenylmethylsulfofluoride (PMSF) and sodium orto-vanadate (final concentrations 1 mM) protease inhibitor cocktail (Cell Signaling Technology, USA, #5871). The samples of 15 µg of protein were mixed with Laemmli loading buffer, containing 1 mM dithiotreitol (Cell Signaling Technology, #7723), denatured in 99 °C for 5 minutes, resolved on 10% gels during SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked in Clear Milk Blocking Buffer™ (Thermo Scientific, USA, #37587) for 1 h in RT. The following primary antibodies (the 1:1000 dilution in blocking buffer) were used: anti-sucrase-isomaltase, rabbit polyclonal (Sigma Aldrich, USA, #SAB2102141); anti-intestinal alkaline phosphatase, mouse monoclonal (Novus Biologicals, USA, #NBP2 37389); anti-villin-1, rabbit polyclonal (Novus Biologicals, #NBP1 85336). To confirm equal loading anti-β-actin mouse monoclonal antibody (1:4000 dilution, Sigma-Aldrich, Germany #A2228) was used. For the signal detection anti-rabbit IgG HRP-linked antibodies (Jackson Immuno Research, USA, #11-035-003), anti-mouse IgG (H+L), HRP conjugated antibodies (Thermo Scientific, USA, #31432) and a chemiluminescent peroxidase substrate Super Signal West Pico Plus™ (Thermo Scientific, USA, #34577) were used. In certain cases, Restore™ stripping buffer (Thermo Scientific, #21059) was used for dissociation of primary antibodies and re-probing. The luminescent signal was recorded using ChemiDoc™ system (Bio-Rad, USA) and the densitometric analysis of the protein bands was performed using ImageJ freeware software (NIH, USA).

Evaluation of mitochondrial polarization

The Caco-2 and HepG2 cells were seeded onto glass-bottom 12-well plates (MatTek Corp., USA) and cultured in the presence of Brussels sprouts digestates for 48 h, prior to the staining with JC-1 dye (Thermo Fisher, USA). JC-1 was dissolved in DMSO (0.5 mg ml^{-1}), and then in warm phenol red-free, serum-free RPMI medium supplemented with 0.1% bovine serum albumin (RPMI-SFM) to final concentration of 10 µg ml^{-1}. The cells were incubated in the dye solution for 15 minutes, then washed 3 times in Ca^{2+}/Mg^{2+} containing PBS and the fresh RPMI-SFM was replaced. JC-1, a membrane permeant dye, possesses different light emission maxima dependent on its aggregation status. In polarized mitochondria with high mitochondrial transmembrane potential (ΔΨm) JC-1 exists in monomers with light emission maximum in red (~590 nm), whereas in depolarized mitochondria JC-1 forms aggregates with light emission maximum in green (~529 nm). The cell culture images were recorded using the inverted fluorescent microscope (Axio Observer, Zeiss, Germany), equipped with optical sectioning *Apotome* structured illumination (confocal like) system and the incubation chamber with the controlled atmosphere (37 °C, 5% CO_2). In all the images the intensity of red and green fluorescence, as well as cell surface area were measured in the individual cells using the ZEN 2012 image analysis software (Zeiss, Germany).

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Amino acid analysis

Amino acid analysis was performed according to the method of Moore and Stein (Moore and Stein 1951, Smith 2003). Lyophilized samples were hydrolyzed in liquid 6M HCl containing 0.5% phenol at 110 °C for 24 hours under an argon atmosphere. The hydrolysates were lyophilized, dissolved in an appropriate volume of dilution buffer (sodium citrate buffer pH 2.2) and filtered through a 0.45 µm syringe filter before applying to the chromatographic amino acid analyzer (AAA400, Ingos, Czech Republic). Amino acids were determined by ion-exchange chromatography, with strong cation ion-exchanger and sodium-citrate elution buffers system followed by post-column derivatization with ninhydrin and spectrophotometric detection at 570 and 440 nm, according to standard protocol of manufacturer. Sulphur-containing amino acids were analyzed as oxidation products obtained by performic acid oxidation followed by standard hydrolysis procedure with HCl. For calibration, the amino acid standard solution was used (Sigma, USA). Evaluation of the acquired data was performed using the software of chromatographic device (Chromulan, Pikron, Czech Republic).

Statistical analysis

The data were collected from between two and five independent experiments, performed in tri- or tetraplicates. The statistical significance (for $p < 0.05$) was tested with one-way ANOVA and Tukey's post-hoc tests (the comparisons among the experimental groups within each cell line), using STATISTICA software (Tibco Software Inc., USA), version 13.3.

Results

The Brussels sprouts *in vitro* digestates up-regulate activity of the enterocyte marker sucrase-isomaltase

Irrespective to the method of hydrothermal processing, we did not detect any signs of cytotoxicity in the Caco-2 and HepG2 cultures treated with the digestates of Brussels sprouts. The digestate of raw Brussels sprouts had a positive effect on Caco-2 cell proliferation, as compared to the digestates from processed vegetables (Fig. 1).

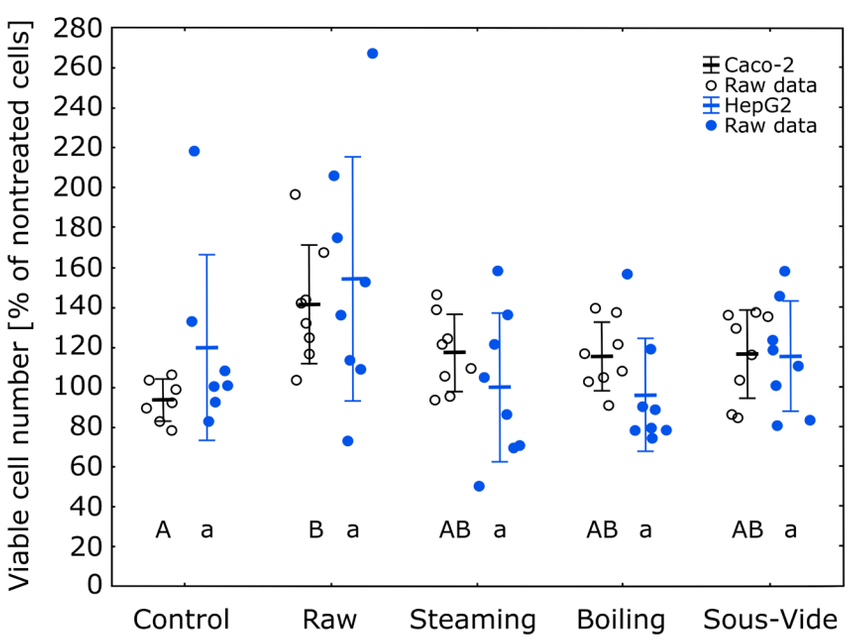

Fig 1. The viable cell number of Caco-2 and HepG-2 after the 48 h incubation with Brussels sprouts *in vitro* digestates. The bars represent percentage of nontreated cells (mean+/-SD). The same letter below the plots indicate lack of statistically significant differences, upper case and lower case letters indicate the results of statistical analysis performed on Caco-2 and HepG2 data, respectively. Control – the cells treated with the supernatant after centrifugation of the digestive enzymes in concentrations used during *in vitro* digestion; nontreated - the cells cultured in the regular medium.

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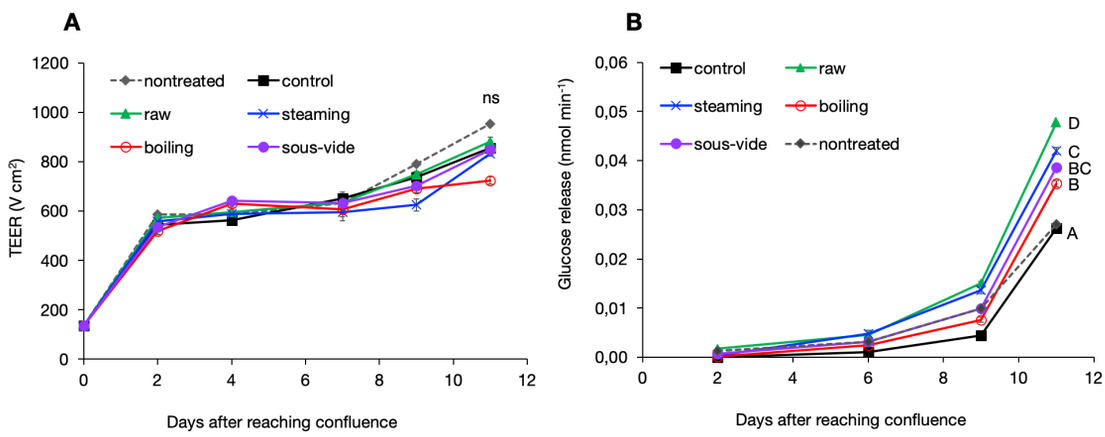
Caco-2 cells cultured as a monolayer of the porous membrane inserts submerged in the cell culture medium undergo differentiation into mature enterocyte-like phenotype. The process is manifested by formation of tight junctions, which are responsible for epithelial barrier function. Development of impermeable cell layer can be monitored by trans-epithelial electric resistance (TEER) measurements, where high TEER values represent a tight epithelial barrier. In our experiments, the digestate from boiled Brussels sprouts achieved lower TEER values, as compared to the other processing methods, but the differences were not statistically significant (Fig. 2A).

The presence of digestates from raw Brussels sprouts positively affected the activity of enterocyte functional marker enzymes SI and ALPI (Fig. 2 BC). The applied cooking methods did not alter ALPI activity in comparison to control, but within the tested experimental groups digestate of raw vegetables showed significantly higher ALPI activity as compared to sous-vide and steaming. Steaming, boiling and sous-vide up-regulated the SI activity as compared to control, but these processing methods had lower impact than raw digestate, which produced the highest stimulation of SI activity (Fig. 2B). The high ALPI and SI activities in case of raw Brussels sprouts corresponded with elevation of their protein levels (Fig. 2D). Sous-vide processing increased the expression of ALPI and SI on the level of protein (Fig. 2D), although this effect did not reflect in exceptionally high activities of these enzymes.



C

Processing method	ALPI activity v_0 [p-NP µmol [l*min^{-1}]]	
NT	6.18±0.59	n=17
Control	4.09±0.36 ^{AB}	n=14
Raw	7.03±1.08 ^{B}	n=13
Steaming	3.25±0.69 ^{A}	n=15
Boiling	4.39±0.78 ^{AB}	n=12
Sous-vide	3.72±0.55 ^{A}	n=18

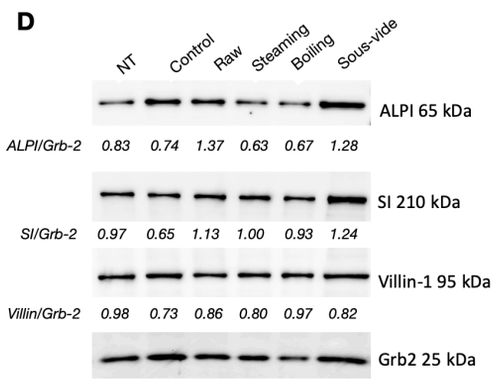


Fig. 2. The differentiation Caco-2 cells into enterocyte-like cells in the presence of the Brussels sprout *in vitro* digestates. (A) Trans-epithelial electric resistance (TEER) of a Caco-2 monolayer (V*cm^2 of cell surface area), points represent mean+/-SD; n=6, ns – nonsignificant differences; (B) Sucrase-isomaltase activity (SI) of Caco-2 cells during the differentiation in the presence of Brussels sprouts digestates (calculated as the rate of glucose release from sucrose [nmol glucose min^{-1}]); the plot shows data from a representative experiment, n=3; (C) intestinal alkaline phosphatase (ALPI) activity of Caco-2 cells differentiated in the presence of Brussels sprouts digestates for 11 days. The enzyme activity is calculated as the initial rate (p-NP µmol [l*min]$^{-1}$), NT – nontreated; (D) The protein levels of the enterocyte differentiation markers: ALPI and SI, the epithelial cell marker Villin-1 and Grb-2 used as a reference protein and loading control. The figure shows the representative immunoblots. The numbers under the protein bands represent the ratio of each evaluated protein band density to the density of a corresponding Grb-2 band. The same letter in superscript indicates the lack of statistically significant differences at $p < 0.05$.

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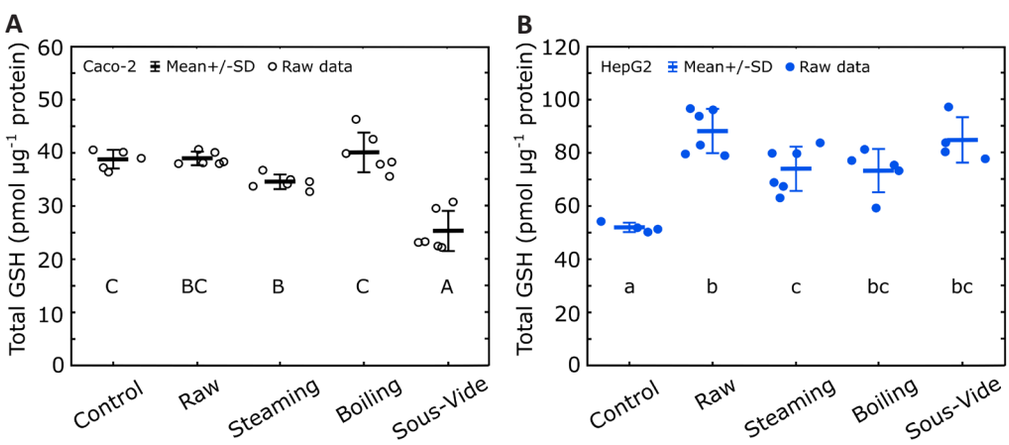
The hydrothermal processing influences the intracellular glutathione level and mitochondrial performance

To evaluate the impact of digestates on the GSH levels and mitochondrial function we have tested not only the intestinal epithelial cells, but also hepatocytes. Hepatocytes are the main target of bioactive compounds absorbed by enterocytes. Food components reach liver through portal vein. We observed the striking difference in the response of two cell lines, Caco-2 and HepG2 in respect to intracellular GSH levels and mitochondrial polarization (Fig. 3). In epithelial cells, sous-vide processing significantly decreased total GSH level in comparison to raw material, which indicates lower health benefits of vegetables cooked that way. We observed that boiling and steaming had the same impact on total GSH as raw vegetables, therefore the shorter hydrothermal treatments are better than sous-vide in terms of GSH storage (Fig. 3A). In HepG2 cells all the digestates increased total GSH level in comparison to control (Fig. 3B).

GSH is synthesized from glycine, glutamate and cysteine, therefore we decided to measure the content of these amino acids in the Brussels sprouts digestates. The rate-limiting substrate for GSH synthesis is cysteine (Bitensky 1990). Cysteine can be replenished via transsulfuration pathway from methionine and serine, therefore both sulfur-containing amino acids are important for the maintenance of GSH level. Glycine, another GSH component, is utilized in multiple metabolic processes and its provision has a positive impact on GSH synthesis (McCarty et al. 2018). The last GSH component, glutamate is usually present in the cells in high concentrations and can be easily restored through glutamine deamination or transamination of α-ketoglutarate, a Krebs cycle intermediate. Cysteine and methionine contents were the highest in the digestates from raw Brussels sprouts, which corresponds with high GSH levels in HepG2 cells (Fig. 3 B). Nevertheless, there is no apparent correlation between GSH and these amino acids in case of the processed vegetables (boiling, steaming and sous-vide) in Caco-2 cells. Interestingly, sous-vide digestates provided significantly more glutamate and glycine as compared to raw vegetables, but this did not reflect in high GSH concentration (Fig. 3 AB).



C

	Amino acid content [g per 100 g], (mean ± SD, n=4)			
	Glu	**Gly**	**Cys**	**Met**
Raw	0.754±0.003$^{\beta}$	0.433±0.003$^{\beta}$	0.019±0.000$^{\beta}$	0,010±0.000$^{\beta}$
Steaming	0.868±0.044$^{\alpha}$	0.493±0.024$^{\alpha}$	0.013±0.002$^{\alpha}$	0,007±0.001$^{\alpha}$
Boiling	0.643±0.026$^{\gamma}$	0.491±0.007$^{\alpha}$	0.012±0.001$^{\alpha}$	0,008±0.001$^{\alpha}$
Sous-vide	0.870±0.013$^{\alpha}$	0.517±0.013$^{\alpha}$	0.015±0.003$^{\alpha\beta}$	0,009±0.002$^{\alpha\beta}$

Fig. 3. The total intracellular glutathione (GSH) concentration in Caco-2 (A) and HepG2 cells (B) cultured in the presence of the Brussels sprouts *in vitro* digestates. The bars represent pmol glutathione/µg protein in the cell lysates (mean+/-SD). (C) The content of the amino acids important for GSH synthesis measured in the Brussels sprout dialysates, expressed as equivalent of amino acids released from the vegetable portion subjected to the *in vitro* digestion (g per 100g). The same letter below the plots or in superscript indicates the lack of statistically significant differences at $p < 0.05$.

In respect to mitochondria, we observed that digestates from the hydrothermal processed Brussels sprouts (steamed, boiled and sous-vide) down-regulated the total cellular mitochondrial load measured as the average sum of green and red JC-1 fluorescence normalized to cell surface area (Fig. 4A). Interestingly, both Caco-2 and-HepG2 showed the same trend in response to the digestates, although the total abundance of mitochondria was higher in hepatocytes, as expected.

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Among the hydrothermal treatments steaming and sous-vide decreased the fraction of polarized mitochondria (red/green ratio), in Caco-2 and HepG2 cells, respectively (Fig. 4B). Unexpectedly, the digestates from boiled vegetables worked much better than steamed in case of Caco-2, but worse in HepG2 cells (Fig. 4B). HepG2 cells responded to the digestate from raw vegetables with significantly elevated fraction of polarized mitochondria, whereas sous-vide decreased the mitochondrial polarization as compared to control (Fig. 4 BC). The digestate from raw Brussels sprouts promoted higher mitochondrial membrane potential in HepG2 cells, whereas in Caco-2 cells it had not changed the polarization as compared to control (Fig. 4 BC). These results indicate that the choice of processing method differentially affects the enterocyte-like cells and hepatocytes.

A

Processing method	Sum of green and red fluorescence			
	Caco-2		*HepG2*	
	n	*mean ± SD*	*n*	*mean ± SD*
NT	381	9.94±0.51	355	24.26±0.9
Control	195	$13.68±0.63^{B}$	172	$20.20±0.95^{c}$
Raw	163	$12.67±0.66^{B}$	188	$16.28±0.84^{b}$
Steaming	161	$9.71±0.56^{A}$	213	$11.16±0.55^{a}$
Boiling	125	$9.72±0.62^{A}$	201	$11.97±0.62^{a}$
Sous - vide	189	$9.93±0.52^{A}$	311	$12.83±0.53^{a}$

B

Processing method	Red/green fluorescence ratio			
	Caco-2		*HepG2*	
	n	*mean ± SD*	*n*	*mean ± SD*
NT	381	0.42±0.02	355	0.40±0.01
Control	195	$0.59±0.04^{B}$	172	$0.27±0.01^{bc}$
Raw	163	$0.50±0.02^{AB}$	188	$0.40±0.01^{d}$
Steaming	161	$0.47±0.02^{A}$	213	$0.29±0.01^{c}$
Boiling	125	$0.59±0.03^{AB}$	201	$0.25±0.01^{b}$
Sous - vide	189	$0.49±0.02^{AB}$	311	$0.19±0.00^{a}$

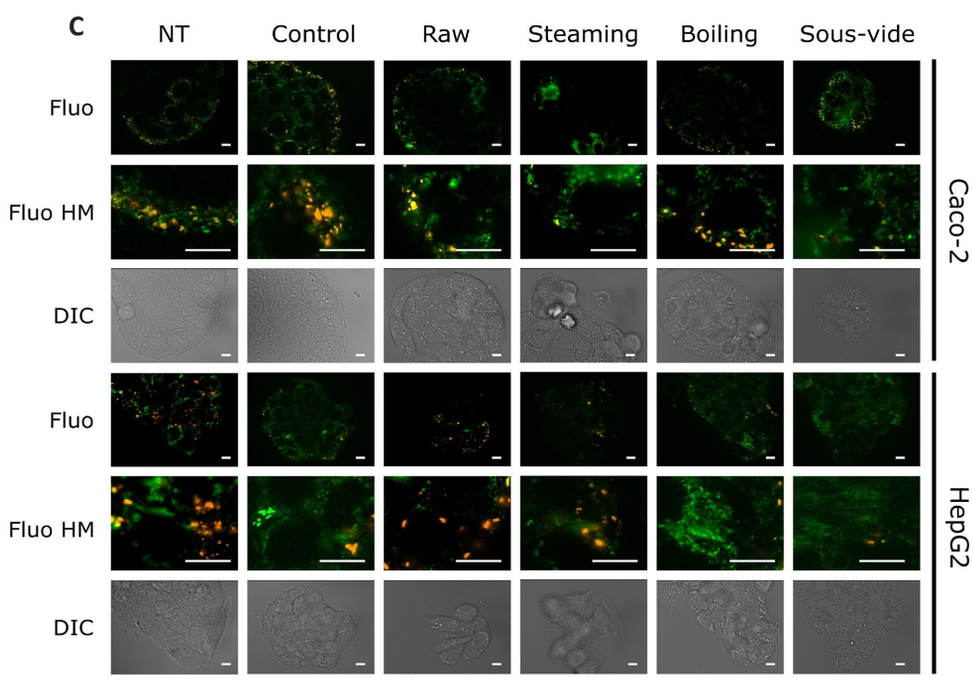

Fig. 4. (A) The total mitochondrial load calculated as the sum of red and green fluorescence intensity in the individual Caco-2 and HepG2 cells stained with JC-1 dye after the incubation with the Brussels sprouts *in vitro* digestates (mean+/-SD). (B) The mitochondrial polarization calculated as the ratio of red (polarized) to green (depolarized) fluorescence intensity in individual cells stained with JC-1 dye after the incubation with the Brussels sprouts digestates (mean+/-SD)**.** NT – nontreated, the same letter in superscript indicates the lack of statistically significant differences at $p < 0.05$. (C) The representative microscopic images (fluorescent and differential interference contrast, DIC) from the Caco-2 and HepG2 cultures treated with the Brussels sprouts digestates and stained with JC-1. Control – the cells treated with the supernatant after centrifugation of the digestive enzymes in concentrations used during in vitro digestion; NT - nontreated, the cells cultured in the regular medium. FLUO HM – high magnification fluorescent microphotographs. Bar: 10 µm.

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Discussion

Our results support the general opinion that hydrothermal processing affects the bioactivity of Brussels sprouts, and the beneficial effects on SI activity and mitochondrial load observed for the *in vitro* digestates from the raw material were not that obvious for the other digestates. Generally, the shorter (7–15 min) processing methods, such as boiling and steaming produced better results in terms of the studied aspects of intestinal epithelium and hepatocyte physiology, as compared to longer procedure, like sous-vide (50 min).

Our earlier studies (Doniec et al. 2022b) focused on differences in the content of selected bioactive compounds in Brussels sprouts subjected to three methods of heat treatment (sous-vide, steaming and boiling) both before and after *in vitro* digestion. The analysis revealed that sous-vide, thanks to vacuum packaging, limiting the contact of the plant material with the water medium and precise temperature control, enabled the preservation of the highest concentration of total polyphenols and ABTS antioxidant activity, even higher than in the raw Brussels sprouts (Doniec et al. 2022b). This result can be explained by the degradation of plant tissues as a result of temperature, increasing the pool of polyphenolic compounds that can be determined and the formation of new antioxidant bundles. However, both the antioxidant capacity towards ABTS and DPPH radicals showed a significant decrease after the *in vitro* digestion (Doniec et al. 2022b).

ITCs derived from cruciferous plants have well-documented chemopreventive activity with selective apoptosis induction in various cancer types, e.g. breast carcinoma (Xiao et al. 2008). The detailed studies on isolated mitochondria revealed that benzyl-isothiocyanate (BITC) elicited mitochondrial damage, loss of the inner membrane potential, blockade of respiration by interfering with electron transfer chain, which lead to oxidative stress and ultimately apoptotic cell death (Nakamura et al. 2002). The mitotoxic activity of ITCs was attributed to their electrophilic nature (Kawakami et al. 2005). However, these studies focused on high concentrations (20–200 μM) of pure ITCs, applied as anti-cancer agents. Our experimental model assumed the range of concentrations of Brussels sprout-derived bioactive components close to physiological situation in a particular dietary treatment. Therefore, as expected, we did not observe cytotoxicity, but rather mild effects on both mitochondrial polarization and GSH stores (Fig. 3 and 4). As far as we are aware, this is the first study that assessed these parameters in the intestinal epithelial and hepatocyte-like cells in the model of Brussels sprouts *in vitro* digestion.

The effect of hydrothermal treatment of Brussels sprouts on the GLS content remains complex. It depends not only on the plant tissue damage, leaching out of bioactive components into aqueous medium, but also on the activity of myrosinase (Florkiewicz et al. 2017). Verkerk (2002) concluded that thermal treatment of *Brassica* vegetables including Brussels sprouts resulted in the 40–70% reduction of GLS content with regards to raw plant material, mostly due to the release of active molecules from plant tissue to water medium and their thermal breakdown. As the GLS level closely correlates with cells glutathione stores, confirmation of these results can be observed in a higher level of intercellular GSH (Fig. 3A) for the raw Brussels sprouts as compared to the heat-processed plants. Moreover, cooking practices strongly alter the GLS–myrosinase system interaction (Ferrarini et al. 2012). One might therefore expect that the vacuum seal used in sous-vide enables a higher retention of GLS than in other cooking methods and consequently a higher intracellular GSH level. Nevertheless, the opposite tendency was observed for sous-vide technique and its negative effect on the GLS content in Brussels sprouts, approximately 40% lower than after boiling (Florkiewicz et al. 2017). Our results support these observations, because the digestates from the boiled sprouts improved significantly GSH stores in both cell lines in comparison to sous-vide (Fig. 3A). Sarvan et al. (2014) observed that GLS released into the medium are more stable that the ones remaining in the Brussels sprout tissue. Our data, showing the lowest GSH levels both in Caco-2 and HepG2 cells treated with the *in vitro* digestates after sous-vide processing, support this notion.

Two factors control the rate of intracellular GSH synthesis: (i) the GCL (EC 6.3.2.2) activity, and (ii) availability of cysteine as a substrate. Both GCL and methionine transmethylation and transsulfuration pathways that restore cysteine are particularly active in liver (Stipanuk 2020, Labarrere and Kassab 2022). That could explain the higher GSH content in HepG2 cells as compared to Caco-2 (Fig. 3). Raw Brussels sprout digestates were the richest source of sulfur amino acids, but steamed and sous-vide processed vegetables provided significantly more glutamate and glycine than raw (Fig. 3B). However, there was no apparent correlation between the content of the mentioned amino acids and the intracellular GSH level. In case HepG2, the cells treated with the digestates from raw and sous-vide processed Brussels sprouts, which had higher cysteine and methionine content than the digestates from boiled and steamed vegetables, exhibited significantly higher GSH levels (Fig. 3B). The weak correlation between the cysteine content in the digestates and the intracellular GSH level in Caco-2 may indicate that the cells had sufficient cysteine provision from the culture media (it was a regular DMEM medium that contained 200 μM

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cysteine), so this component was no longer a limiting one. The difference in the cell behavior between Caco-2 and HepG2 might be attributed to the characteristic of hepatocyte physiology and their higher natural GSH synthesis rate (Labarrere and Kassab 2022).

So far, mostly negative influence of food processing on the amount of amino acids was demonstrated in Brussels sprouts, broccoli, kale and white and green cauliflower (Murcia et al. 2001, Lisiewska et al. 2008, Slupski et al. 2009, Slupski et al. 2010, Korus 2012). However, these reports focused on analyses of amino acid content in plant material, which was not digested *in vitro*. Our study estimates the amounts of amino acids available for absorption in the model of intestinal epithelia and further hepatocytes. In general, the digested Brussels sprouts is a richer source of all the four tested amino acids than broccoli (Murcia et al. 2001). In comparison to kale or white and green cauliflower, the Brussels sprouts digestates provided more glutamate and glycine, but contained less sulfur amino acids (Lisiewska et al. 2008, Slupski et al. 2009, Slupski et al. 2010, Korus 2012). Noteworthy, in the cited reports the analyses were performed on raw, cooked, blanched or frozen vegetables, without *in vitro* digestion.

The negative impact of hydrothermal treatment on the health-promoting effect of Brussels sprouts, as in the case of GSH, reflects also in the lower mitochondrial polarization. This result is not surprising, because the proper mitochondrial function strongly correlates with the reduction of intracellular oxidative stress, for which a GSH sufficient supply is needed. Ferrarini et al. (2012) analyzed the chemoprotective effects of raw and cooked Brussels sprouts on the oxidative damage of DNA in HT-29 cells and found that correlation between high antioxidant activity and chemopreventive action on the redox status disappeared after hydrothermal treatment (boiling, steaming and microwaved), in contrast to raw plant material. Bearing in mind that Brussels sprouts are mainly consumed after thermal processing and considering the preservation of its strong pro-health values, it is crucial to look for methods of thermal processing other than those analyzed in this work. New approaches to culinary processing might improve the degree of retention of health-beneficial compounds at a level comparable to that of a raw vegetable.

Conclusions

Raw Brussels sprouts can have superior effects on the intestinal epithelial cell differentiation and mitochondrial performance. However, hydrothermal processing is a necessary step during the preparation of this vegetable for the consumption. Our data suggest that the processing should be as short as possible to minimally interfere with the biologically active components of plant tissue, so short boiling and steaming seems to preserve the bioactivity of the *in vitro* digestates better than longer processing, such as sous-vide. In terms of protection from oxidative stress, boiling retains glutathione stores and mitochondrial polarization in the epithelial cells to the same extent as in case of the digestates from raw Brussels sprouts. Mainly negative in relation to boiling, results of research on the composition of Brussels sprouts are explained by the high degree of leaching of compounds from plant tissue. Looking from a different perspective, our results indicating a better effect of boiling in relation to mitochondrial polarization and GSH level may result from excessive leaching compared to other techniques. During the boiling not only nutrient and bioactive compounds are lost in water, but plant tissues also lose compounds that reduce and hinder the absorption and assimilation of nutrients, thereby improving absorption of remaining compounds by the intestinal epithelial cells. Regarding the aim to preserve the health-promoting properties of Brussels sprouts, there is a need for evaluation of other processing methods, apart from those used in this work.

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References

Becker, T.M. & Juvik, J.A. 2016. The role of glucosinolate hydrolysis products from brassica vegetable consumption in inducing antioxidant activity and reducing cancer incidence. Diseases 4. https://doi.org/10.3390/diseases4020022

Bitensky, L. 1990. Glutathione: Chemical, biochemical, and medical aspects. In: Dolphin, D., Poulson, R. & and Avramović, O. (eds). New York: Wiley-interscience. Part a. Cell Biochemistry and Function 8: 139–139. https://doi.org/10.1002/cbf.290080215

Bogaards, J.J., Verhagen, H., Willems, M.I., van Poppel, G. & van Bladeren, P.J. 1994. Consumption of brussels sprouts results in elevated alpha-class glutathione s-transferase levels in human blood plasma. Carcinogenesis 15: 1073–1075. https://doi.org/10.1093/carcin/15.5.1073

Cartea, M.E. & Velasco, P. 2008. Glucosinolates in brassica foods: Bioavailability in food and significance for human health. Phytochemistry Reviews 7: 213–229. https://doi.org/10.1007/s11101-007-9072-2

Agricultural and Food Science (2023) 32: xx–xx
https://doi.org/10.23986/afsci.130131

Dinkova-Kostova, A.T. & Kostov, R.V. 2012. Glucosinolates and isothiocyanates in health and disease. Trends in Molecular Medicine 18: 337–347. https://doi.org/10.1016/j.molmed.2012.04.003

Doniec, J., Florkiewicz, A., Dulinski, R. & Filipiak-Florkiewicz, A. 2022a. Impact of hydrothermal treatments on nutritional value and mineral bioaccessibility of brussels sprouts (*Brassica oleracea* var. Gemmifera). Molecules 27. https://doi.org/10.3390/molecules27061861

Doniec, J., Florkiewicz, A., Dziadek, K. & Filipiak-Florkiewicz, A. 2022b. Hydrothermal treatment effect on antioxidant activity and polyphenols concentration and profile of brussels sprouts (*Brassica oleracea* var. Gemmifera) in an *in vitro* simulated gastrointestinal digestion model. Antioxidants (Basel) 11. https://doi.org/10.3390/antiox11030446

Ernst, I.M., Wagner, A.E., Schuemann, C., Storm, N., Hoppner, W., Doring, F., Stocker, A. & Rimbach, G. 2011. Allyl-, butyl- and phenylethyl-isothiocyanate activate nrf2 in cultured fibroblasts. Pharmacological Research 63: 233–240. https://doi.org/10.1016/j.phrs.2010.11.005

Ferrarini, L., Pellegrini, N., Mazzeo, T., Miglio, C., Galati, S., Milano, F., Rossi, C. & Buschini, A. 2012. Anti-proliferative activity and chemoprotective effects towards DNA oxidative damage of fresh and cooked brassicaceae. British Journal of Nutrition 107: 1324–1332. https://doi.org/10.1017/S0007114511004272

Ferruzza, S., Rossi, C., Scarino, M.L. & Sambuy, Y. 2012. A protocol for in situ enzyme assays to assess the differentiation of human intestinal caco-2 cells. Toxicology In Vitro 26: 1247–1251. https://doi.org/10.1016/j.tiv.2011.11.007

Florkiewicz, A., Ciska, E., Filipiak-Florkiewicz, A. & Topolska, K. 2017. Comparison of sous-vide methods and traditional hydrothermal treatment on gls content in brassica vegetables. European Food Research and Technology 243: 1507–1517. https://doi.org/10.1007/s00217-017-2860-z

Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.A., Chevolleau, S., Gasc, N., Tulliez, J. & Terce, F. 2000. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in ht29 human colon cancer cells. Cancer Research 60: 1426–1433.

Grabacka, M.M., Gawin, M. & Pierzchalska, M. 2014. Phytochemical modulators of mitochondria: The search for chemopreventive agents and supportive therapeutics. Pharmaceuticals (Basel, Switzerland) 7: 913–942. https://doi.org/10.3390/ph7090913

Hidalgo, A., Ferraretto, A., De Noni, I., Bottani, M., Cattaneo, S., Galli, S. & Brandolini, A. 2018. Bioactive compounds and antioxidant properties of pseudocereals-enriched water biscuits and their *in vitro* digestates. Food Chemistry 240: 799–807. https://doi.org/10.1016/j.foodchem.2017.08.014

Indo, H.P., Yen, H.C., Nakanishi, I., Matsumoto, K., Tamura, M., Nagano, Y., Matsui, H., Gusev, O., Cornette, R., Okuda, T., Minamiyama, Y., Ichikawa, H., Suenaga, S., Oki, M., Sato, T., Ozawa, T., Clair, D. K. & Majima, H.J. 2015. A mitochondrial superoxide theory for oxidative stress diseases and aging. Journal of Clinical Biochemistry and Nutrition 56: 1–7. https://doi.org/10.3164/jcbn.14-42

Johnson, T.L., Dinkova-Kostova, A.T. & Fahey, J.W. 2015. Glucosinolates from the brassica vegetables and their health effects. Encyclopedia of Food and Health. Elsevier Inc.: p. 248–255. https://doi.org/10.1016/B978-0-12-384947-2.00354-8

Kawakami, M., Harada, N., Hiratsuka, M., Kawai, K. & Nakamura, Y. 2005. Dietary isothiocyanates modify mitochondrial functions through their electrophilic reaction. Bioscience, Biotechnology, and Biochemistry 69: 2439–2444. https://doi.org/10.1271/bbb.69.2439

Korus, A. 2012. Effect of technological processing and preservation method on amino acid content and protein quality in kale (*Brassica oleracea* l. Var. Acephala) leaves. Journal of the Science of Food and Agriculture 92: 618–625. https://doi.org/10.1002/jsfa.4619

Labarrere, C.A. & Kassab, G.S. 2022. Glutathione: A samsonian life-sustaining small molecule that protects against oxidative stress, ageing and damaging inflammation. Frontiers in Nutrition 9. https://doi.org/10.3389/fnut.2022.1007816

Lafarga, T., Bobo, G., Vinas, I., Zudaire, L., Simo, J. & Aguilo-Aguayo, I. 2018. Steaming and sous-vide: Effects on antioxidant activity, vitamin c, and total phenolic content of brassica vegetables. International Journal of Gastronomy and Food Science 13: 134–139. https://doi.org/10.1016/j.ijgfs.2018.05.007

Lisiewska, Z., Kmiecik, W. & Korus, A. 2008. The amino acid composition of kale (*Brassica oleracea* l. Var. Acephala), fresh and after culinary and technological processing. Food Chemistry 108: 642–648. https://doi.org/10.1016/j.foodchem.2007.11.030

McCarty, M.F., O'Keefe, J.H. & DiNicolantonio, J.J. 2018. Dietary glycine is rate-limiting for glutathione synthesis and may have broad potential for health protection. Ochsner Journal 18: 81–87.

Messer, M. & Dahlqvist, A. 1966. A one-step ultramicro method for the assay of intestinal disaccharidases. Analytical Biochemistry 14: 376–392. https://doi.org/10.1016/0003-2697(66)90280-6

Mitsiogianni, M., Koutsidis, G., Mavroudis, N., Trafalis, D. T., Botaitis, S., Franco, R., Zoumpourlis, V., Amery, T., Galanis, A., Pappa, A. & Panayiotidis, M.I. 2019. The role of isothiocyanates as cancer chemo-preventive, chemo-therapeutic and anti-melanoma agents. Antioxidants (Basel) 8. https://doi.org/10.3390/antiox8040106

Moore, S. & Stein, W.H. 1951. Chromatography of amino acids on sulfonated polystyrene resins. Journal of Biological Chemistry 192: 663–681. https://doi.org/10.1021/ac60139a005

Murcia, M.A., Lopez-Ayerra, B., Martinez-Tome, M. & Garcia-Carmona, F. 2001. Effect of industrial processing on amino acid content of broccoli. Journal of the Science of Food and Agriculture 81: 1299–1305. https://doi.org/10.1002/jsfa.942

Nakamura, Y., Kawakami, M., Yoshihiro, A., Miyoshi, N., Ohigashi, H., Kawai, K., Osawa, T. & Uchida, K. 2002. Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. Journal of Biological Chemistry 277: 8492–8499. https://doi.org/10.1074/jbc.M109760200

Sanlier, N. & Guler Saban, M. 2018. The benefits of brassica vegetables on human health. Journal of Human Health Research 1: 104.

Sarvan, I., Verkerk, R., van Boekel, M. & Dekker, M. 2014. Comparison of the degradation and leaching kinetics of glucosinolates during processing of four brassicaceae (broccoli, red cabbage, white cabbage, brussels sprouts). Innovative Food Science & Emerging Technologies 25: 58–66. https://doi.org/10.1016/j.ifset.2014.01.007

Agricultural and Food Science (2023) 32: xx–xx
https://doi.org/10.23986/afsci.130131

Slupski, J., Bernas, E., Kmiecik, W. & Lisiewska, Z. 2009. Evaluation of the amino acid content and the quality of protein in florets of white cauliflower: Raw, cooked, and prepared for consumption after freezing. International Journal of Food Science and Technology 44: 629–634. https://doi.org/10.1111/j.1365-2621.2008.01884.x

Slupski, J., Lisiewska, Z., Kmiecik, W., Gebczynski, P. & Sobczynska, L. 2010. The effect of processing on the amino acid content in green cauliflower. Agricultural and Food Science 19: 136–143. https://doi.org/10.2137/145960610791542316

Smith, A.J. 2003. Post column amino acid analysis. In: Smith, B.J. & Totowa, N.J. (eds.). Methods in molecular biology 211: 133–141. https://doi.org/10.1385/1-59259-342-9:133

Staack, R., Kingston, S., Wallig, M.A. & Jeffery, E.H. 1998. A comparison of the individual and collective effects of four glucosinolate breakdown products from brussels sprouts on induction of detoxification enzymes. Toxicology and Applied Pharmacology 149: 17–23. https://doi.org/10.1006/taap.1997.8340

Starzyńska-Janiszewska, A., Duliński, R., Stodolak, B., Mickowska, B. & Wikiera, A. 2016. Prolonged tempe-type fermentation in order to improve bioactive potential and nutritional parameters of quinoa seeds. Journal of Cereal Science 71: 116–121. https://doi.org/10.1016/j.jcs.2016.08.001

Stipanuk, M.H. 2020. Metabolism of sulfur-containing amino acids: How the body copes with excess methionine, cysteine, and sulfide. Journal of Nutrition 150: 2494s–2505s. https://doi.org/10.1093/jn/nxaa094

Suppipat, K., Park, C.S., Shen, Y., Zhu, X. & Lacorazza, H.D. 2012. Sulforaphane induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells. PloS One 7: e51251. https://doi.org/10.1371/journal.pone.0051251

Verkerk, R. 2002. Evaluation of glucosinolate levels throughout the production chain of Brassica vegetables towards a novel predictive modelling approach. [internal PhD, WU, Wageningen University]. Wageningen Universiteit. https://edepot.wur.nl/198988

Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., De Schrijver, R., Hansen, M., Gerhauser, C., Mithen, R. & Dekker, M. 2009. Glucosinolates in brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. Molecular Nutrition & Food Research 53 Suppl 2: S219. https://doi.org/10.1002/mnfr.200800065

Xiao, D., Powolny, A.A. & Singh, S.V. 2008. Benzyl isothiocyanate targets mitochondrial respiratory chain to trigger reactive oxygen species-dependent apoptosis in human breast cancer cells. Journal of Biological Chemistry 283: 30151–30163. https://doi.org/10.1074/jbc.M802529200

Ye, L. & Zhang, Y. 2001. Total intracellular accumulation levels of dietary isothiocyanates determine their activity in elevation of cellular glutathione and induction of phase 2 detoxification enzymes. Carcinogenesis 22: 1987–1992. https://doi.org/10.1093/carcin/22.12.1987

Zyla, K., Ledoux, D.R., Garcia, A. & Veum, T.L. 1995. An *in vitro* procedure for studying enzymic dephosphorylation of phytate in maize-soyabean feeds for turkey poults. British Journal of Nutrition 74: 3–17. https://doi.org/10.1079/BJN19950102