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Thermosonication of Broccoli Florets Prior to Fermentation Increases Bioactive Components in Fermented Broccoli Puree

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Abstract: The aim of this study was to compare the effects of thermosonication (18 kHz at 60 °C for 7 min) pre-treatment with thermal treatment alone (60 °C for 7 min) of broccoli florets prior to pureeing and fermentation on selected bioactive components of fermented broccoli puree. Both thermal and thermosoncation pre-treatments significantly increased the rate of acidification of broccoli puree compared to control untreated broccoli puree, with the time to reach pH 4 being 8.25, 9.9, and 24 h, respectively, for thermally treated, thermosonicated, and control samples. The highest sulforaphane yield of 7268 µmol/kg dry weight (DW) was observed in the thermosonicated samples, followed by 6227 µmol/kg DW and 3180 µmol/kg DW in the thermally treated and untreated samples, respectively. The measurable residual glucoraphanin content was 1642 µmol/kg DW, 1187 µmol/kg DW, and 1047 µmol/kg DW, respectively, in the thermonsonicated, thermally pre-treated, and control fermented samples, indicating that pre-treatment specially by thermosonication increases the extractability of glucoraphanin. The higher sulforaphane yield in the thermosonicated and thermally pre-treated samples could be due to increased extractability and accessibility of glucoraphanin and interaction with myrosinase in addition to the inactivation of epthiospecifier protein (ESP), which directs conversion away from sulforaphane into sulforaphane nitrile.

Keywords: thermosonication; broccoli puree; fermentation; glucoraphanin; sulforaphane; phenolic compounds

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1. Introduction

Fermentation is traditionally used for improving the safety and shelf-life of perishable food materials. Nevertheless, fermentation has several benefits in addition to preservation such as enhancing the nutritional and bioactive attributes of food products [1]. Fermented plant-based products in general are characterised by desirable nutritional properties, improved digestibility of macronutrients, a reduced content of anti-nutritional factors, and a high level of bioactive compounds [2]. Fermented plant-based products can also be used as alternative vehicles to fermented dairy products for delivery of probiotic microorganisms for vegans and consumers with allergies and intolerances to dairy products.

Brassica vegetables such as broccoli and cabbage have recently attracted significant attention mainly because of their high content of nutrients and phytochemicals. For instance, broccoli contains high levels of glucosinolates (GLs), phenolic compounds, carotenoids, vitamins, and essential minerals [3]. Glucoraphanin (GR, 4-methylsulfinylbutenyl glucosinolate) is the most abundant GL in broccoli, comprising 81% of the broccoli total GL content [4]. GR is converted by the activity of myrosinase (thioglucoside glucohydrolase EC 3.2.1.147) into sulforaphane (SF: $C_6H_{11}NOS_2$) [5], the best-known Brassica-derived bioactives with several potential health benefits, or sulforaphane nitrile (SFN, 5-methylsulfinylpentane nitrile), its non-bioactive counterpart. The relative conversion to these two metabolites is

dependent on temperature, pH, and the presence of a proteinaceous co-factor Epthiospecifier protein (ESP) that modulates the conversion reaction [6]. In the presence of active ESP, sulforaphane nitrile is the main product of conversion. Sulforaphane is associated with several beneficial health effects, such induction of phase II detoxification enzymes in anti-carcinogenic metabolism [7], inhibitory effects on cancer cell proliferation, antihypertensive and cardioprotective effects, and as a supplementary treatment for type 2 diabetes [8].

The conversion of glucoraphanin occurs when broccoli tissue is damaged during mechanical processing (e.g., cutting and/or chewing), since myrosinase and glucosinolates are present in different cell compartments in the intact plant. In raw broccoli, the main product of conversion during such processes is sulforaphane nitrile due to the active ESP present in the broccoli matrix. Several approaches have been assessed to enhance sulforaphane formation in broccoli products such as mild heat treatment to selectively inactivate ESP while maintaining myrosinase activity [9], high pressure thermal processing [10], lactic acid fermentation [11], and a combination of mild heat with lactic acid fermentation [12]. For instance, mild heating to inactivate ESP combined with fermentation enabled a ≈16-time increase in sulforaphane yield in broccoli puree compared to non-treated broccoli puree, which was attributed to the selective inactivation of ESP and fermentation-induced increase in the accessibility of glucoraphanin for myrosinase-catalysed conversion into sulforaphane. Recently, Shokri et al. [13] introduced mild heat combined with ultrasound (thermosonication) as an alternative method to improve the release and bio-accessibility of GR in broccoli, facilitating its enzymatic conversion into SF compared to mild heat treatment alone. Several studies indicate that ultrasound pre-treatment of food substrates alone or in combination with mild heat enhance fermentation processes via enhanced tissue permeabilisation and release of endogenous enzymes, conformational changes of enzymes enabling exposure of more active sites for interaction with their substrates, and chemical and physical modification of substrates providing an optimal environment for starter cultures and enzyme activities [14].

Against this background, it was hypothesised that thermosonication pre-treatment of broccoli prior to lactic acid fermentation would enhance GL release from broccoli matrix and facilitate its myrosinase-catalysed hydrolysis to sulforaphane. This study compared the effects of thermal treatment (60 °C for 7 min) of broccoli florets with that of thermosonication (18 kHz at 60 °C for 7 min) on the extractable GR, sulforaphane, and total polyphenolic content of broccoli puree after lactic acid fermentation. The microbial qualities of the fermented broccoli purees were also assessed.

2. Materials and Methods

2.1. Sample Preparation

Fresh broccoli was purchased from a local supermarket in Werribee, Australia, and kept at 4 °C prior to use. Broccoli heads were thoroughly washed with sterile Milli-Q water, and the florets were cut into 2–3 cm length below the crown. Afterwards, a ratio of 75 g broccoli florets to 250 mL sterile Milli-Q was prepared in a 500 mL glass container for thermal or thermosonication pre-treatments. This broccoli to water ratio was chosen on the basis of our earlier study on thermosonication pre-treatment of broccoli florets, where a higher ratio led to inhomogeneous acoustic energy densities delivered to the samples, and a lower ratio resulted in dilution of samples for adequate sulforaphane production [13].

2.2. Thermal and Thermosonication Pre-Treatments

Thermal pre-treatments were performed by placing the glass containers containing the broccoli–water mixture in a water bath maintained at 60 °C. The treatment was conducted for 7 min. The thermosonication (TS) pre-treatment was performed using an 18 kHz ultrasonic processor (UP500S, 500 W, Hielscher Ultrasonics GmbH, Teltow, Germany) coupled with a titanium sonotrode (tip diameter 18 mm, length 70 mm, submerged ≈3 cm into the samples) at an ultrasonic power density of 0.41 ± 0.02 W/g for 7 min at 60 °C

as described by Shokri et al. [13]. In order to maintain the temperature of samples at the target experimental temperature during TS treatment, samples were placed into a chamber with a water circulation system. For both thermal and TS pre-treatments, timing started after the samples reached the experimental temperature. Immediately after thermal and TS treatments, samples were cooled in ice-water and were pureed as described below for fermentation experiments. The thermal and TS treatment conditions were selected on the basis of our previous study [13]. All experiments were performed in triplicate.

2.3. Fermentation Starter Culture Preparation

The starter culture consortium consisted of one *Lactobacillus plantarum* (B1) strain and two *Leuconostoc mesenteroides* (BF1, BF2) strains that were previously isolated from broccoli leaves and broccoli florets, respectively, and identified by 16S RNA sequencing at the Australian Genome Research Facility (AGRF) Ltd. (Melbourne, Australia) [11]. The seed cultures, which were stored at −80 °C, were thawed and activated by inoculation into 10 mL of MRS broth (Oxoid, VIC, Australia) and incubated aerobically at 30 °C for 24 h. Two millilitres of each medium containing activated cultures were then inoculated into 200 mL of MRS broth. After incubation of the broth at 30 °C for 24 h, the cells were harvested by centrifugation (3500× *g* for 15 min at 4 °C), washed twice with sterile phosphate-buffered saline (PBS), and resuspended in sterile Milli-Q water at a biomass of 10 Log CFU/mL. The *Leuconostoc mesenteroides* (BF1 and BF2) cell suspensions were then mixed at a volumetric ratio of 1:1, and then one part of the BF1–BF2 mixture was mixed to one part of the *L. plantarum* cell suspension to form the starter culture consortium. The starter culture was stored with 15% *v*/*v* glycerol at −80 °C until use for broccoli fermentation experiments.

2.4. Fermentation

Thermal or TS pre-treated and untreated (as control) broccoli florets (with 250 mL water added to 75 g florets), as described above, were pureed using a magic bullet kitchen blender (Nutribullet pro 900 series, LLC, Capital brands, los Angeles, CA, USA) for 1 min and were transferred into 500 mL sterile plastic containers for fermentation experiments. The starter cultures, which were stored at −80 °C, were used for inoculating the broccoli puree samples (325 g) after thawing, washed twice with sterile PBS, and resuspended in Milli-Q water. The culture dosage was $\approx 10^8$ CFU per gram of broccoli puree. The samples were incubated in water bath maintained at 30 °C until the pH reached less than 4.0. Changes in pH values during fermentation were recorded using a pH-data logger (MM-PIT-4U, EA Instruments, Wembley, UK). After fermentation was completed, sample aliquots were aseptically taken for microbiological analysis, and the remaining sample was stored at −20 °C for physicochemical and phytochemical analyses. All experiments were repeated three times.

2.5. Determination of pH and Titratable Acidity

The pH of samples was determined using a pH meter (PHM240, MeterLab, Terni, Italy) after dilution with Milli-Q water to a 1:1 *w*/*v* ratio. Titratable acidity (TA) measurement was conducted as described in [10]. Accordingly, 10 mL of diluted samples (1:1 *w*/*v* with Milli-Q water) were titrated using 0.1 M NaOH to the end point pH = 8.1 using an automatic titrator (Titralab 854 titration manager, Radiometric Analytical, Lyon, France), and TA was calculated as follows:

$$\text{TA (gram equivalent of lactic acid per litre)} = \frac{\text{v} \times \text{acid factor} \times 1000}{\text{sample volume}} \quad (1)$$

where v is titer volume of NaOH. The acid factor for lactic acid is 0.009.

2.6. Microbiological Analysis

Lactic acid bacteria, Enterobacteriaceae, and yeast and mould counts were determined prior to and immediately after fermentation using appropriate media as described in [11]. Briefly, 1:10 serial dilutions of broccoli suspensions were prepared using a solution of 9.5 g/L of sterilised maximum recovery diluent (MRD) (Oxoid, VIC, Australia).

Lactic acid bacteria were counted using a surface plate method by plating 0.1 mL of each diluted sample on MRS agar in duplicate and incubation in an anaerobic jar with anaerobic sachets (Thermo Fisher Scientific, Carlsbad, CA, USA) for 48 h at 30 °C. Enterobacteriaceae counts were determined using the pour plate method by mixing 1 mL of each sample with violet red bile glucose (VRBG) agar in duplicate and incubation for 24 h at 37 °C. For yeast and mould counts, 0.1 mL of each sample was plated on potato dextrose agar (PDA) with the pH adjusted to 3.5 using 10% tartaric acid in duplicate and incubated for 5 days at 25 °C. Following incubation, the colony-forming units were counted, and the results were expressed as log CFU/mL.

2.7. Glucoraphanin Analysis

Four grams of sample slurry were well mixed with 25 mL hot 70% methanol (70 °C) and then blended using an Ultra-Turrax T25 (JANKE and KUNKEL; IKA Labortechnik, Staufen, Germany) for one minute at 13,500 rpm. The resultant suspension was incubated at 70 °C for 30 min in a shaking water bath (Thermoline, Wetherill Park, NSW, Australia) with continuous shaking at 100 rpm. Afterward, samples were cooled in ice-water and centrifuged at 5000× *g* for 15 min at 4 °C, the supernatants were collected, and the precipitate was re-extracted under the same conditions a second time. Finally, the extracts were combined and evaporated to dryness using vacuum spin dryer (SC250EXP, Thermo Fisher Scientific, Carlsbad, CA, USA) at ≈22 °C. The residues were kept at −20 °C until HPLC analysis. The GR content was quantified on the basis of the method of Cai et al. [11] with some modifications. Briefly, before HPLC analysis, the extracts were dissolved in 1 mL acetonitrile/water (85:15, *v*/*v*) with 30 mM ammonium formate and filtered through a 0.22 µm membrane filter (Merck Millipore, Billerica, MA, USA). An Alliance HPLC instrument (Waters Corporation, Milford, MA, USA) equipped with a Photo Diode Array Detector 2998 was used for analysis, and separation was carried out on a Luna® 3 µM Hydrophilic Interaction Liquid Chromatography (HILIC) 200 °A column (100 × 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 85:15 *v*/*v* acetonitrile/water containing 30 mM ammonium formate as eluent A and 100% acetonitrile as eluent B. An isocratic flow program of 70% eluent A and 30% eluent B was used at a flow rate of 2.0 mL/min with an injection volume of 100 µL at a constant column temperature of 25 °C for 8 min elution time at a detection wavelength of 235 nm. GR was quantified by a calibration curve from the glucoraphanin standard (Cayman Chemical Company, Ann Arbor, MI, USA), and results were shown in µmol/kg dry weight (DW) of broccoli.

2.8. Sulforaphane Analysis

2.8.1. Extraction

SF extraction from the puree was conducted according to the method described in [11]. In brief, 10 mL Milli-Q water was added to 4 g of frozen broccoli puree samples and vortexed for 1 min, followed by adding 20 mL of ethyl acetate, sonication for 5 min using a sonication bath (IDK Technology Pty Ltd., Mooroolbark, VIC, Australia), and shaking for 20 min at 4 °C. The mixtures were then centrifuged at 5000× *g* for 15 min at 10 °C, the supernatants were collected, and the precipitates were re-extracted with 15 mL ethyl acetate. The supernatants of from the extraction steps were pooled together and dried using a vacuum spin dryer (SC250EXP, Thermo Fisher Scientific, Carlsbad, CA, USA) at ambient temperature (≈22 °C).

2.8.2. Solid Phase Extraction

During broccoli fermentation, a variety of compounds are generated which can interfere with SF chromatographic separation and analysis. The extracts, therefore, were cleaned using a selective extraction and separation solid phase extraction (SPE) method established by Han and Row [15] with modification to remove possible interferences prior to the chromatographic analysis. The dried crude extracts were reconstituted in 1 mL 100% acetonitrile by vortexing for 1 min, followed by 5 min sonication. A 500 µL aliquot of each reconstituted sample was loaded onto a SPE column (55 µm × 70 A° silica column in a 3 mL tube, Phenomenex Inc., Torrance, CA, USA), which was previously conditioned with 4 mL methyl tert-butyl ether (Sigma-Aldrich, St. Louis, MO, USA), followed by washing the columns with 4 mL ethyl acetate and eluting SF with 4 mL of ethanol under vacuum. Ethanol then was removed from the eluents using a vacuum spin dryer (SC250EXP, Thermo Fisher Scientific, Waltham, MA, USA). The dried samples were dissolved in 1 mL of 30% acetonitrile, filtered using a 0.22 µm membrane filter (Merk Millipore, Billerica, MA, USA), and stored at −20 °C until analysis.

2.8.3. UPLC Analysis

For the quantitative analysis of SF, an Acquity™ Ultra Performance LC systems (Waters Corporation, Milford, MA, USA) managed by Acquity console software (Waters Corporation, Milford, MA, USA) was used according to the method established by Cai et al. [11]. The SF separation was carried out on a 2.1 × 50 mm, Acquity BEH C18 column using 0.1% formic acid in Milli-Q water as eluent A and 0.1% formic acid in acetonitrile as eluent B. A gradient flow program started with 10% eluent B for the first 2 min, increasing to 20% eluent B over the next 3 min, and then decreasing to 10% of eluent B over the subsequent 5 min. A flow rate of 0.350 mL/min was used for an injection volume of 5 µL at a constant column temperature of 30 °C. Quantification was achieved using a calibration curve prepared with SF standard (DL–sulforaphane, Sigma-Aldrich, St. Louis, MO, USA), and results were expressed as µmol/kg DW of broccoli.

2.9. Total Phenolic Content

The total phenolic contents (TPC) were estimated using the Folin–Ciocalteau method as described by Singleton and Rossi [16] with some modifications. In brief, the frozen samples were freeze-dried, and 50 mg of freeze-dried samples were suspended in 10 mL of acidified (1% HCl) methanol/water (70:30, v/v), followed by sonication for 8 min. The suspension was shaken overnight (≈18 h) at 4 °C in a flask shaker, and was centrifuged at 25,000× g for 10 min at 4 °C. The supernatants were collected, and the precipitates were re-extracted using the same solvent. The supernatants from the two extractions were pooled together and were then filtered through a 0.2 µm filter. A total of 100 µL of each filtrate was mixed well with 500 µL of 0.2 N Folin–Ciocalteu reagent, and after 3 min incubation at room temperature, 400 µL of sodium carbonate solution (7.5% w/v in Milli-Q water) was added and vortexed for 15 s. The resultant mixture was incubated at 37 °C for 60 min, and thereafter its absorbance was measured at 765 nm. The TPC was calculated using a calibration curve developed using gallic acid standard, and the results were expressed as milligrams gallic acid equivalent (GAE) per gram dry weight (mg GAE/g DW).

2.10. Data Analysis

The effects of the thermal and TS pre-treatments on microbial and phytochemical attributes of fermented broccoli puree were statistically evaluated by one-way analysis of variance (ANOVA) using SPSS software (version 18.0 for Windows, SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used in the data analysis since the experimental design involved three independent treatments. The differences among the means of the three independent experiments were compared using Duncan's post hoc test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1. pH and Titratable Acidity

3.1.1. Prior to Fermentation

The initial TA and pH of broccoli puree without pre-treatment were 4.30 g/L and 6.82, respectively. Thermal or thermosonication (TS) pre-treatments did not have significant ($p > 0.05$) effects on TA or pH of broccoli puree (Table 1). Others reported no significant changes in TA or pH in purple cactus pear (*Opuntia ficus indica*) juice [17], tangerine juice [18], and camu–camu nectars [19] after thermosonication treatment.

Table 1. Effects of thermal or thermosonication treatments of broccoli florets on changes in pH and titratable acidity in broccoli purees before and after lactic acid fermentation.

		pH	Acidity (g/L)
Before fermentation	Control	6.84 ± 0.02 [a]	4.30 ± 0.12 [a]
	Thermal pre-treated	6.76 ± 0.05 [a]	4.18 ± 0.02 [a]
	TS pre-treated	6.79 ± 0.03 [a]	4.08 ± 0.10 [a]
After fermentation	Control	3.80 ± 0.01 [b]	10.9 ± 0.27 [b]
	Thermal pre-treated	3.65 ± 0.02 [c]	11.7 ± 0.59 [c]
	TS pre-treated	3.87 ± 0.01 [d]	13.1 ± 0.69 [d]

Amounts followed by different lower case superscript letters within the same column denote significant differences ($p < 0.05$). Reported values are mean ± SD. 'Control' denotes non-pre-treated broccoli puree.

3.1.2. Effects on Fermentation

When the broccoli purees made from untreated broccoli florets or pre-treated florets were fermented with a mixed-culture (*Leuconostoc mesenteroides* (BF1, BF2; 1:1) and *Lactobacillus plantarum* (B1)), the end point of the fermentation (pH 4) was reached after 24.1 h for control untreated samples and at shorter times of 8.25 and 9.9 h for thermal and TS pre-treatments, respectively. These results can be explained by heat- and sonication-induced cell rupture and modification of the structure of the plant matrix, increasing the physical accessibility of fermentable substrates (e.g., sugars and other nutrient sources), resulting in a higher acidification rate [20]. These results are in agreement with those reported by Shokri, Terefe [21] who observed a lower pH and a higher TA during ultrasound-assisted (23 kHz, 10 µm amplitude for 3 min and 5 min) fermentation by *Lactobacilluse brevis* (LMG 6906) compared to those in control without ultrasound treatments.

3.2. Microbial Analysis

3.2.1. Prior to Fermentation

The initial counts of LAB, Enterobacteriaceae, and yeast and mould in control broccoli puree samples were 3.85, 3.47, and 3.44 Log CFU/g, respectively, which are relatively lower than those previously reported for raw broccoli by Cai, Wang [11]. Differences may be expected due to agronomic conditions and varietal differences in broccoli as well as post-harvest handling and storage conditions of the broccoli prior to use. The initial LAB count decreased by 0.61 and 1.04 Log CFU/g following thermal (60 °C for 7 min) and TS (18 kHz at 60 °C for 7 min) treatments, respectively. This is consistent with previous reports that ultrasound waves increase the sensitivity of microorganisms to heat due to the cavitation phenomenon which causes damage to the cell wall and the membrane of the microorganisms, increasing microbial lethality [18]. Similar results were reported by Alcántara-Zavala, de Dios Figueroa-Cárdenas [22], Alvarado-Morales, Minjares-Fuentes [23], and Alves, dos Santos [17].

With regard to the Enterobacteriaceae, the initial count was 3.47 log CFU/g and decreased to 2.6 and 2.26 log CFU/g after thermal and TS treatments, respectively. Similar to LAB, ultrasound treatment slightly enhanced the thermal inactivation of Enterobacteriaceae. Thermal and TS sensitivity of Enterobacteriaceae has been reported in several previous studies. Alves, dos Santos [18] reported thermosonication at 35 kHz at 60 °C/5 min, and

60 °C/10 min resulted in a non-detectable level of total plate counts, moulds, and yeasts, and total and thermotolerant coliforms.

In contrast to the effects of pre-treatments on LAB and Enterobacteriaceae, TS treatment did not alter total yeast and mould count, while thermal treatment led to a 0.69 log CFU/g decrease. Although the amount of yeast and mould can be decreased due to ultrasound-induced cellular damages, sono-physical effects can break the cell clusters, resulting in a higher or no change in cell count by plate count agar enumeration [21,24,25]. These results indicate that the plate count method may not present the actual cell count following TS or ultrasound treatments. Therefore, alternative microbial counting should be considered.

3.2.2. After Fermentation

Despite a decrease in original LAB count immediately after both pre-treatments, a significant ($p < 0.05$) increase to maximum counts of 9.16 and 9.45 Log CFU/g after fermentation was observed in thermal and TS pre-treated samples, respectively (Table 2). These counts were significantly higher ($p < 0.05$) than those in control sample (7.99 Log CFU/g). This observation suggests that thermal or TS pre-treatment modification to the material and accessibility of fermentable substrates results in a faster LAB proliferation [24,25].

Table 2. Effects of thermal or thermosonication treatments of broccoli florets on microbial load of broccoli purees before and after lactic acid fermentation.

		Microbial Counts (Log CFU/mL)		
		LAB	**Enterobacteriaceae**	**Yeast and Mould**
Before fermentation [1]	Control	3.85 ± 0.04^{a}	3.47 ± 0.04^{a}	3.46 ± 0.02^{a}
	Thermally pre-treated	3.25 ± 0.03^{b}	2.60 ± 0.09^{b}	2.77 ± 0.13^{b}
	TS pre-treated	2.81 ± 0.05^{c}	2.26 ± 0.12^{c}	3.42 ± 0.02^{c}
After fermentation	Control	7.99 ± 0.04^{d}	ND [2]	ND
	Thermally pre-treated	9.16 ± 0.02^{e}	ND	ND
	TS pre-treated	9.45 ± 0.02^{f}	ND	ND

Entries followed by different lower case superscript letters (a–f) within the same column denote significant differences ($p < 0.05$). Reported values are mean ± SD. 'Control' denotes non-pre-treated broccoli puree. [1] Before inoculation with lactic acid bacteria culture. [2] ND means not detected.

Enterobacteriaceae was not detected in the fermented samples. Similarly, Cai, Wang [11] reported no detectable level of Enterobacteriaceae in broccoli puree after lactic acid fermentation. Similarly, yeast and mould were not detected in the fermented samples (Table 2). It is known that antibacterial activities of enzymatic hydrolysates of glucosinolates such as isothiocyanates in broccoli puree and LAB metabolites such as organic acids, bacteriocins, and antifungal peptides inhibit the growth of food spoilage and pathogenic organisms [26].

3.3. Glucoraphanin Content

3.3.1. Prior to Fermentation

The initial measurable content of GR in the non-processed broccoli floret was 3149.35 µmol/kg DW, which is slightly lower than the GR levels of 4.4–16.4 µmol/g DW reported by Matusheski, Juvik [9]. The measurable GR content dramatically decreased from 3149.35 to 689, 813, and 1241 µmol/kg DW, respectively, in the control, pre-heated, and thermosonicated broccoli puree samples (Figure 1A), most likely due to myrosinase-catalyzed conversion. In intact broccoli tissue, myrosinase and its substrate glucoraphanin are in separate compartments, and hence there is no myrosinase-catalyzed conversion in intact broccoli. During pureeing and thermophysical treatments, tissue decompartmentalisation occurs, which leads to myrosinase–glucoraphanin interaction and conversion of glucoraphanin to its metabolites (sulforaphane and sulforaphane nitrile, depending on the presence of active ESP and the pH of the matrix) [6,9,12]. The GR content in thermal or TS pre-treated purees were significantly higher ($p < 0.05$) than those in control puree (Figure 1A). This could have been due to heat- and ultrasound-induced structural mod-

ification of the broccoli matrix, facilitating the release of potentially bound GR [4,12,13], counteracting the effects of myrosinase-catalyzed conversion. A higher level of GR in thermally and TS pre-treated broccoli puree samples compared to non-pre-treated broccoli puree was also observed in our previous study [13]. A relatively higher GR content was observed in the TS pre-treated samples compared the thermally pre-treated samples. This can be attributed to an enhanced level of cell wall and plant matrix disruption due to the extreme shear forces generated during acoustic cavitation, which facilitate the release of plant constituents [27]. These results are in agreement with those observed by Aguilar-Camacho, Welti-Chanes [28], who reported a 795% increase in GR content in broccoli florets following ultrasound treatment compared to control samples. Pongmalai, Devahastin [29] also reported an 87% increase in GR extractability in steamed white cabbage using combined ultrasound and microwave-assisted extractions.

3.3.2. Post Fermentation

All broccoli purees showed a significant ($p < 0.05$) increase in GR after fermentation. The contents of GR were 1047, 1187, and 1642 μmol/kg DW in the control, thermally pre-treated, and TS pre-treated fermented broccoli purees, respectively, despite a significant conversion to sulforaphane (Section 3.4). Ye, Huang [30] also reported a significant increase in different GLs after LAB fermentation, where GR increased from trace level in the autoclaved broccoli to 29–237 μg/g DW after fermentation depending on the LAB strain used for broccoli puree fermentation. The increased GR content may be attributed to cell disruption and degradation by enzymes produced by lactic acid bacteria [31], which may increase the extractability-bound phytochemicals including GR from the broccoli matrix. A higher residual level of GR was observed in pre-treated fermented samples counteracting the effects of enzymatic conversion, indicating that both thermosonication and thermal treatment increase the extractability and accessibility of glucoraphanin in synergy with lactic acid fermentation.

3.4. Sulforaphane Yield

3.4.1. Prior to Fermentation

The effects of thermal or TS pre-treatments on SF content of fermented broccoli purees are presented in Figure 1B. Thermal and TS pre-treatments of broccoli floret increased the SF yield from 943 μmol/kg DW in un-pre-treated (control) broccoli puree to 2160 and 2346 μmol/kg DW in thermal and TS pre-treated samples, respectively (Figure 1B). The initial amount of 943 μmol/kg DW in un-pre-treated broccoli puree was significantly higher than those reported by Sarvan, Kramer [32] and Jones, Frisina [33], who observed the SF content in fresh broccoli floret were 2.96 μmol/100 g FW (≈296 μmol/kg DW) and 14.9–76.2 mg/kg DW (84.0 to 429.8 μmol/kg DW), respectively. The higher SF content in broccoli puree could be related to SF formation during and after broccoli floret pureeing, although the purees were frozen immediately after production. The amount of SF in thermal pre-treatment samples was 129% higher than those in the control. Similar effects of thermal treatment on SF yield have been reported in other studies. For example, Ghawi, Methven [34] reported a six-time increase in SF yield in broccoli floret after 8 and 12 min of thermal processing at 70 °C. A higher level of SF formation in thermally processed broccoli is mainly attributed to the selective thermal inactivation of the epithiospecifier protein (ESP), a non-catalytic cofactor for myrosinase that directs GR conversion to sulforaphane nitrile instead of SF [9]. The heat-induced increase in the extractability and accessibility of GR [35], which was also observed in this study (Section 3.3), may also have contributed to the observed higher yield of SF in mild-heat-treated broccoli.

Thermosonication pretreatment (18 kHz at 60 °C for 7 min) enhanced SF yield over and above the effects of heating alone, with a 149% and 8.6% higher SF yield compared to untreated and thermally pre-treated broccoli purees, respectively. The positive effect of TS pre-treatments of broccoli floret on SF formation in broccoli puree was also observed in our previous study [13]. This could have been due to release of bound GR or induced

cell disruption due to acoustic cavitation that facilitates the GR release and accessibility for myrosinase, as well as the inactivation of ESP.

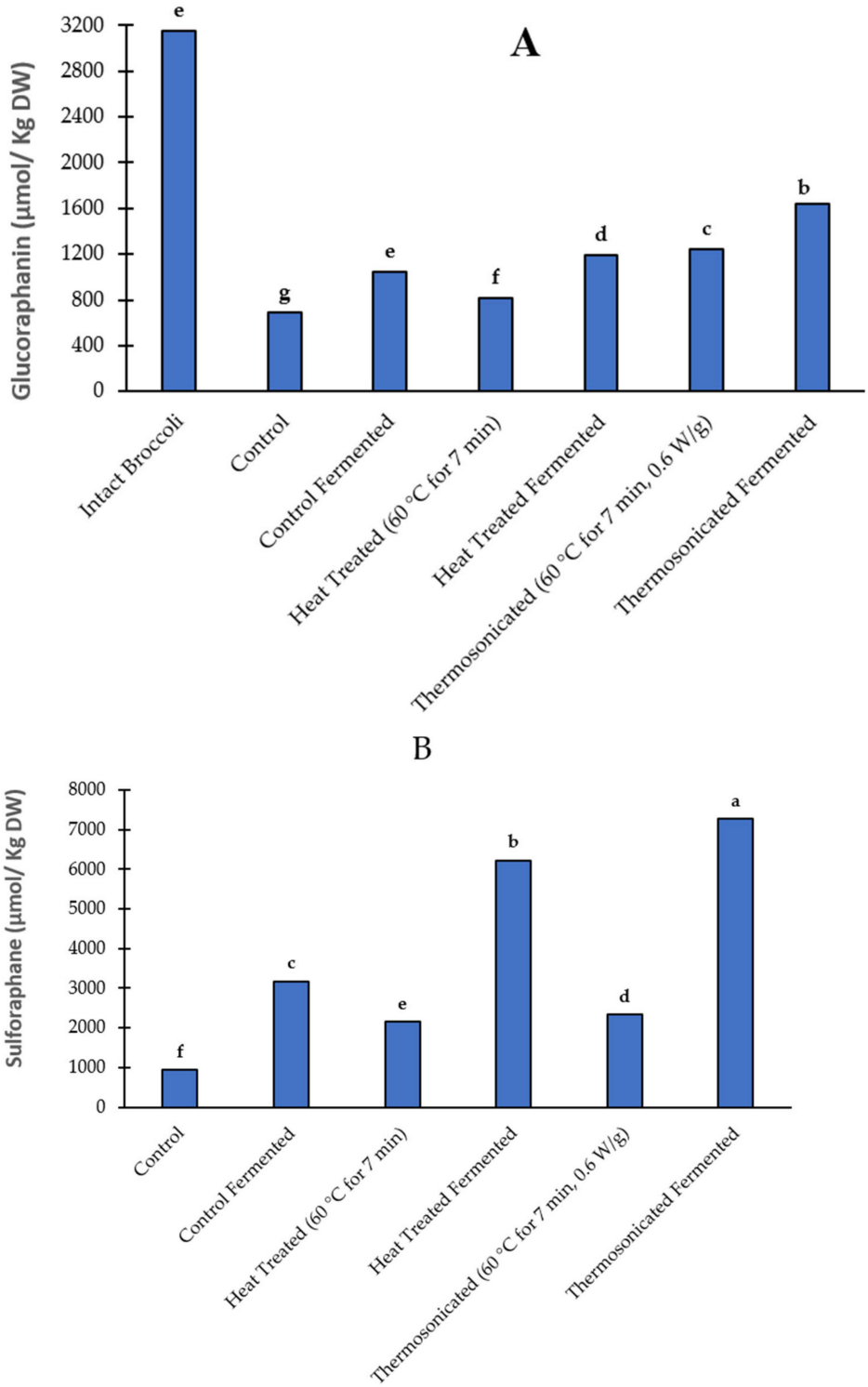

Figure 1. Effects of thermal or thermosonication treatments of broccoli florets on glucoraphanin (**A**) and sulforaphane (**B**) content of broccoli purees before and after lactic acid fermentation. 'Control' denotes non-pre-treated broccoli puree. Different lowercase letters (a to f) over the bars denote significant differences ($p < 0.05$).

3.4.2. Post Fermentation

Lactic acid fermentation studies of fruit and vegetables have indicated these matrices are suitable substrate that support growth and metabolic activities of starter cultures that enhance the nutritional profile of such substrates through the synthesis of vitamins and bioactive compounds [36]. Earlier studies indicate that biomass pre-treatment could improve the fermentation process, resulting in a fermented product with better nutritional characteristics [20]. In this sense, pre-treated broccoli purees were fermented with a mixed culture consisting of *Leuconostoc mesenteroides* (BF1, BF2; 1:1) and *Lactobacillus plantarum* (B1) to evaluate the effects of thermal or TS pre-treatments on SF yield in fermented broccoli puree.

The SF content of untreated (control) samples increased to 3180 µmol/kg DW from an initial value of 943 µmol/kg DW prior to fermentation. Similarly, the SF content in pre-treated samples increased significantly to 6227 µmol/kg DW in thermally pre-treated samples and 7268 µmol/kg DW in TS pre-treated samples. These results indicate that fermentation is a promising method to achieve higher SF yield in broccoli puree and that this also can be amplified using thermal pre-treatment or a combination of thermal pre-treatment with ultrasound. A contributory factor to the observed increase during fermentation could be possible increase in cell disruption and improved accessibility of GR due to the activity of the starter culture. Lactic acid bacteria (LAB) can potentially degrade the broccoli cell compartment using their polysaccharide-degrading enzymes such as cellulases and pectinases [31], resulting in an enhanced accessibility of GR. An enhanced extractability of GR after lactic acid fermentation was reported by Ye, Huang [30] who observed an increase in GR from trace amount to 66–540 µmol/kg DW in broccoli puree with no active myrosinase. In addition to the starter culture, the microbiota originally present in broccoli may also contribute to the enhanced SF formation during fermentation. A myrosinase-like activity has been described in several lactic acid bacteria [37–39], although the cultures that were used in this study do not possess myrosinase activity [29]. Similar results were observed by Cai, Wang [11], who reported an increased yield of SF from 845 µmol/kg DW in broccoli puree to 1617 µmol/kg DW in fermented puree after LAB fermentation. In another study, a ≈16 times increase in SF yield was observed in broccoli puree subjected to in-pack heat treatment (65 °C per 3 min) followed by LAB fermentation, which remained stable (≈94% retention) even after two weeks of storage at 4 °C [11]. Peñas, Pihlava [40] also observed a significant increase in the volatile glucosinolate hydrolysis compounds of different white cabbage cultivars including iberin, iberin nitrile, allyl cyanide, allyl isothiocyanate, and sulforaphane following LAB fermentation.

3.5. Total Phenolic Content

3.5.1. Prior to Fermentation

The untreated broccoli puree samples had a total phenolic compound content (TPC) of 4.21 mg GA/g DW, which increased significantly ($p < 0.05$) to 4.38 and 4.91 mg GA/g DW after thermal and TS pre-treatments, respectively (Figure 2). The effects of heating on increasing TPC has also been reported for purple cactus pear (*Opuntia ficus indica*) juice [16] and broccoli floret [41]. Turkmen, Sari [42] also reported a significant increase in TPC in broccoli following heating, which was related to increased levels of free phenolics due to thermal cleavage of inter-molecular interactions between phenolics. Gliszczyńska-Świgło, Ciska [43] and Şengül, Yildiz [44] also reported a similar increase in TPC of broccoli after heat treatment. A further increase of 12.1% in TPC was observed in freeze-dried broccoli powders using a combination of thermal and ultrasound pre-treatments, consistent with the results reported for tangerine juice [18], cactus pear juice [45], apple juice [46], and purple cactus pear (*Opuntia ficus indica*) juice [16]. As the phenolic compounds are either in soluble form in the vacuoles or bound to other cell structures such as cellulose, pectin, hemicellulose, proteins, and lignin in the cell wall [47], it is possible that physical and chemical effects due to the collapse caused by ultrasound cavitation disrupt the linkages between the phenolic compounds and plant matrix, releasing these compounds [48].

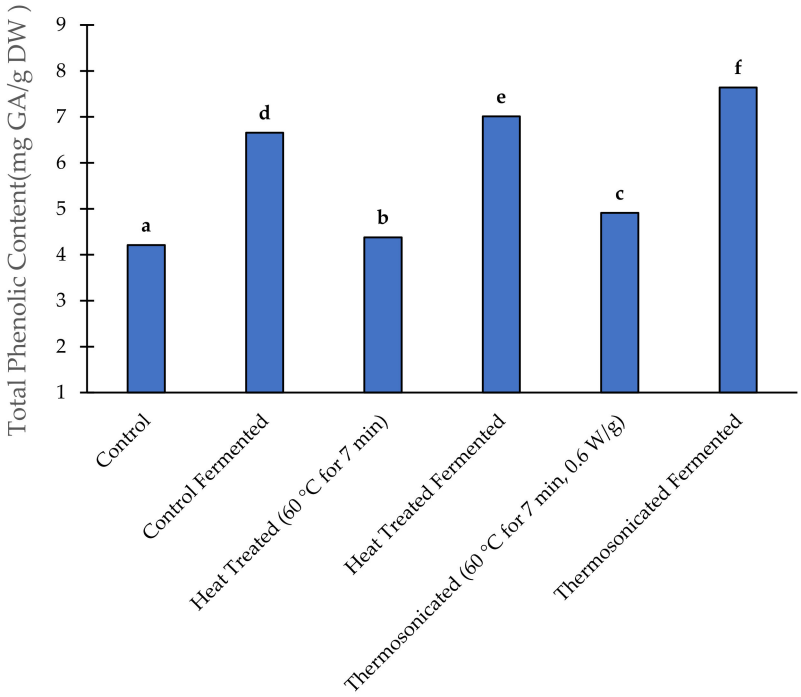

Figure 2. The changes in the total phenolic content of broccoli purees made from thermally or thermosonication-treated broccoli florets before and after lactic acid fermentation. 'Control' denotes non-pre-treated broccoli puree. Different lowercase letters (a–f) over the bars denote significant differences ($p < 0.05$).

3.5.2. After Fermentation

The TPC of broccoli puree samples further increased after fermentation in both untreated (control) and pre-treated broccoli purees. The highest amount of 7.64 mg GA/g DW was obtained in TS pre-treated samples followed by thermally pre-treated and control samples with TPC content of 7.01 and 6.65 mg GA/g DW, respectively. Similar increases in TPC following fermentation were reported for soy milk [48], broccoli puree [11,30,49], and an orange juice–milk-based beverage [50]. Cai, Wang [11] reported a significant increase of ≈83% of TPC in broccoli puree after fermentation from 15.7 to 28.7 mg GA/g DW. Ye, Huang [30] also reported a significant increase in the 10 major phenolic acids in broccoli from 289 µg total phenolics/g DW to 903–3105 µg/g DW in LAB-fermented broccoli puree. The increase in TPC can be attributed to various factors such as release of bound polyphenols or conversion of complex polyphenols to smaller catabolites with higher antioxidant activity and contribution to the measured TPC. Some LAB produce cell-wall-polysaccharide-degrading enzymes such as cellulases and pectinases, resulting in the release of bound polyphenolic compounds and higher TPC [51]. Cleavage of protein–polyphenol complexes by microbial proteases may also lead to the release of polyphenols after fermentation [52]. In addition, LAB produce a range of enzymes such as glycosidases, tanases, and esterases that convert phenolic glycosides, tannins, and phenolic acid esters into aglycones and phenolic acids [53] with higher antioxidant capacity increasing TPC. It has to be noted that the Folin–Ciocalteu TPC assay is not a direct measure of the total polyphenol content in a food matrix but rather a measure of the total antioxidant activity and electron-donating compounds in the matrix [54]. The lower pH of the fermented samples may also have contributed to their higher TPC, since the stability of phenolic compounds is dependent on pH [55], with lower pH improving TPC stability.

4. Conclusions

Thermal and thermosonication (TS) pre-treatment of broccoli floret led to a faster acidifying rate during broccoli puree fermentation with a mixed starter culture consisting

of *Leuconostoc mesenteroides* (BF1, BF2; 1:1) and *Lactobacillus plantarum* (B1). The TS-assisted fermentation also significantly increased the sulforaphane yield in broccoli puree ferments compared to control and thermally pre-treated samples. Moreover, the fermented TS pre-treated broccoli puree resulted in the highest improvement in total polyphenol content compared to control and thermally pre-treated samples. TS-assisted fermentation is a promising route to manufacture fermented broccoli purees enriched with sulforaphane and phenolic compounds with potential health benefits.

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