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Absorption and Excretion of Glucosinolates and Isothiocyanates after Ingestion of Broccoli (*Brassica oleracea* L. var *italica*) Leaf Flour in Mice: A Preliminary Study

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Abstract: During the harvesting of the broccoli plant, the leaves are discarded, being considered a by-product that may be up to 47% of total broccoli biomass, representing a large amount of wasted material. The use of broccoli leaves is of great interest in the sense that this wasted material is rich in health promoter compounds, such as isothiocyanates. In this study, C57BL/6J mice ingested 790 mg/kg broccoli leaf flour, and the presence of glucosinolates and isothiocyanates in the plasma, liver, kidney, adipose tissue, faeces and urine was analysed at 1, 2, 4, 8, 12 and 24 h post-ingestion. In plasma, only glucoerucin (GE), glucobrassicin (GB), sulforaphane (SFN) and indol-3-carbinol (I3C) were detected, and all four compounds peaked between 4 and 8 h after ingestion. The compounds SFN, SFN-glutathione (SFN-GSH), SFN–cysteine (SFN-CYS) and SFN-N-acetyl-cysteine (SFN-NAC) were excreted in faeces at high levels, while glucoraphanin (GR), the precursor of SFN, was not detected in any biological samples other than urine. In the liver, the compounds GE, SFN-CYS, SFN-NAC and I3C were detected, while in the kidney, only GE, GB and SFN-GSH were present. None of the glucosinolates and isothiocyanates analysed were detected in fat tissue. These results demonstrate that glucosinolates and their derivatives were absorbed into the bloodstream and were bioavailable after ingestion of powdered broccoli leaves.

Keywords: broccoli by-products; bioavailability; health; C57BL/6J mice

1. Introduction

In recent years, the scientific community has focused on valuing the by-products resulting from the agro-industrial sector [1]. Broccoli (*Brassica oleracea* L. var. *italica*) is one of the most cultivated crops worldwide [2], thus having a great economic and environmental impact in terms of the generation of by-products [3–5]. Broccoli leaves are usually discarded and, depending on the cultivar and growth conditions, they may be up to 47% of the biomass (fresh weight) of a broccoli plant, representing a large amount of wasted material [6]. Nevertheless, their high content of bioactive molecules such as glucosinolates [7,8] make them interesting and suitable for use in functional foods such as cakes, breads, snacks

or beverages [8–12]. Glucosinolates are present in *Brassica* plants and are converted to their respective isothiocyanates by the enzyme myrosinase, which is released after plant tissue damage or by microbial metabolism in the gut [13–15]. In broccoli plants, glucoiberin (GI), glucobrassicin (GB), glucoraphanin (GR) and its reduced form glucoerucin (GE) are some of the glucosinolates present [16]. Isothiocyanates are health promoters as evidenced by several studies, showing potential therapeutic actions in many types of cancer, skin disorders, respiratory conditions, behavioural disorders, cardiovascular disease, diabetes and obesity [8,17–28]. Sulforaphane (SFN) is an isothiocyanate that results from the hydrolysis of GR. SFN is conjugated with glutathione (GSH) to form SFN-GSH, which is subsequently metabolised to SFN-cysteine (SFN-CYS) via the mercapturic acid pathway, culminating in the formation of the final metabolite SFN-N-acetyl-cysteine (SFN-NAC) [29,30]. The isothiocyanate erucin (the sulphide analogue of SFN) can be formed either through GE hydrolysis or through the reduction of the sulfoxide group in SFN [29,31], while iberin is formed after hydrolysis of GI [16]. Both erucin and iberin are also metabolised via the mercapturic acid pathway [31–33]. GB is the precursor of the isothiocyanate indol-3-carbinol (I3C), which under acidic conditions is converted mainly to 3,3′-diindolylmethane (DIM), which does not undergo metabolisation [34,35]. Considering the health benefits reported in the literature for these metabolites, it is important to study their formation and bioavailability.

The inflorescence and broccoli sprouts are generally used in bioavailability studies addressing the compounds present in the broccoli plant. The inflorescence is the part of the plant normally consumed and the broccoli sprouts are quite rich in glucosinolates [36,37]. However, the assessment of the bioavailability of these compounds after ingestion of by-products, such as leaves or stems, also needs to be evaluated. This is especially important in a circular economy context, in which the use of agricultural residues potentially rich in compounds beneficial to health becomes more prominent. Data related with the bioavailability of broccoli leaf main compounds are scarce. Therefore, in the present study, we proceed to quantify the aforementioned glucosinolates and isothiocyanates in plasma, liver, kidney, adipose tissue, faeces and urine from C57BL/6J mice following ingestion of a high dose (790 mg/kg) of broccoli leaf flour (BLF).

2. Materials and Methods

2.1. Broccoli Leaf Processing

Broccoli leaves used in the study were obtained from broccoli (*Brassica oleracea* L. var. *italica* cv. Naxus) plants harvested in July 2019. Plants were acquired from the producer Quinta do Celão, Unipessoal Lda, from a crop field located in Quinta do Celão, Adémia de Baixo, Coimbra, Portugal. After harvesting the plants, the leaves were cut and frozen at −80 °C until processing. Intact plant material was then freeze-dried and ground into flour in a regular food processor.

2.2. Establishment of the Dose of Interest

A person (60 kg) who consumes 75 g of fresh broccoli per meal was considered for the calculations [38]. According to our data, broccoli relative water content was 87.3%; thus, 9.5 g of 75 g corresponds to dry weight (dw). The consumption of 9.5 g dw per person (60 kg) corresponds to a dose of 158.3 mg/kg, rounded to 158 mg/kg. Considering a mouse with a body weight of 25 g, the dose will correspond to 3.95 mg dw/mouse. However, a 5-fold higher dose was used (19.75 mg per 25 g mouse), which corresponds to 790 mg/kg BLF, to ensure the detection of the compounds in a pilot study.

2.3. Animals

Six-week-old male C57BL/6J mice (n = 21; Charles River Laboratories, Saint Germain Nuelles, France) were housed in open cages (n = 3 per cage) with corn cob bedding and environmental enrichment (cardboard rolls). All animals had ad libitum access to a standard diet and tap water. Animals were housed under controlled temperature (21 ± 2 °C), relative humidity (50 ± 10%) and 12 h light/12 h dark cycle. Mice were equally divided into seven

different time points (n = 3). The voluntary oral delivery of the BLF by mice was achieved by the incorporation of BLF into gelatine pellets of unflavoured gelatine with 10% sucrose as a vehicle. All animal experiments were approved by the Animal Welfare and Ethical Review Body of University of Trás-os-Montes and Alto Douro (UTAD) and by the national competent authority Direção-Geral de Alimentação e Veterinária (DGAV, Lisbon, Portugal; license nº 8776).

2.4. Gelatine Ingestion Training

Voluntary drug ingestion is an alternative to more stressful administration methods in animals, such as intragastric gavage, and can be performed using gelatine pellets. However, the animals need a period of training to become used to ingesting the gelatine pellet [39–42]. Thus, after two weeks of acclimatation, the animals were trained to consume gelatine pellets for 5 days. Unflavoured gelatine with 10% sucrose and with 10% corn starch was used for training. The animals were fasted for 16 h before the first contact with the gelatine pellets to encourage them to overcome neophobia [43]. The animals were placed individually in cages and a gelatine pellet (vehicle) was put in each cage. The procedure was repeated on the following days, but without fasting. The animals consumed the gelatine pellets within 10 min, returning immediately to the group's cage after ingestion.

2.5. Administration of Broccoli Leaf Flour

The unflavoured gelatine solution with 10% sucrose served as a vehicle for the ingestion of BLF, based on a previous study by our group [44]. Gelatine solution was prepared at once with the required amount of BLF for all animals, taking into account the average body weight [44]. For the preparation, the required amount of BLF for all animals was divided in two halves, and each half was mixed with 5 mL of the gelatine solution in round-bottomed cups and stored at −20 °C to solidify. Later, each half was divided into 16 portions, corresponding to one dose per animal. The animals were fasted for 16 h before the gelatine administration with the BLF. The animals were placed individually in cages and a gelatine pellet was put in each cage. One hour after ingesting the gelatine pellet, animals were returned to their home cages where they were maintained in the same conditions including access to food and water. Animals were sacrificed at 1, 2, 4, 8, 12 and 24 h after ingestion. The control (CTR) group (designated as t = 0 h) was sacrificed after the ingestion of the gelatine pellets without the BLF, to be subjected to the same stimulus and stress of manipulation.

2.6. Collection of Biological Samples

At the end of each time point, the animals were sacrificed by an overdose of 145 mg/kg sodium pentobarbital (intraperitoneally; EUTHASOL® 400 mg/mL, ESTEVE, Barcelona, Spain), followed by cardiac puncture. Blood was collected in lithium-heparin tubes and then centrifuged (1400× g, 15 min, 4 °C) to obtain plasma [7]. Whenever possible, urine was collected directly from the bladder. Faecal pellets were collected from the large intestine. The kidneys, liver and epididymal adipose tissue were also harvested. Samples were stored at −80 °C before being processed for the determination of glucosinolates and their derivatives. For histopathological analysis, one kidney, one liver lobe and one lung were harvested.

2.7. Histopathology

The organs collected for histopathological analysis were fixed by immersion in 10% buffered formalin [7]. After fixation, organs were sectioned, dehydrated through a graded series of alcohols and embedded in paraffin. Tissue sections with 3 µm thick were stained with hematoxylin and eosin (H&E) and observed under a light microscope for histological analysis.

2.8. Determination of Glucosinolates and Derivatives in Plasma

2.8.1. Pre-Processing Method

The deep-frozen plasma samples were lyophilised and then suspended in 0.2 mL ammonium acetate 13 Mm pH 7/0.1% formic acid in acetonitrile (50:50, *v*/*v*) by vortex and sonication for 10 min. Then, the samples were vortexed and centrifuged (10,000× rpm, 5 min, 4 °C). The supernatants were collected and filtered through a 0.22 µm Millex-HV13 membrane (Millipore Corp., Bedford, MA, USA).

2.8.2. Reverse Phase—High-Performance Liquid Chromatography—Diode Array Detector (RP-HPLC-DAD) Analysis

The RP-HPLC-DAD system (Thermo Finnigan, San Jose, CA, USA) analysis was carried out to determine glucosinolates and their metabolic derivatives in plasma. The analysis equipment is composed of three parts, including an LC pump (Surveyor; Thermo Fisher Scientific, Waltham, MA, USA), autosampler (Surveyor) and PDA detector (Surveyor). Sample extracts, in triplicate, were injected into a C18 column (250 × 4.6 mm, 5 µm particle size; ACE, Aberdeen, Scotland), through a chromatographic gradient developed by applying different percentages of the solvents (A) 13 mM ammonium acetate pH 7 and (B) acetonitrile/formic acid (99.99:0.01, *v*/*v*) (Supplementary Table S1) according to the multipurpose method, which separates intact glucosinolates, isothiocyanates, and their metabolites [45]. The injection volume was 10 µL and the flow rate was kept at 1.0 mL min^{-1}. The glucosinolates and isothiocyanates content of the plasma samples analysed was expressed as µmol/L.

2.9. Determination of Glucosinolates and Derivatives in Urine, Faeces, Kidney, Liver and Adipose Tissue

2.9.1. Pre-Processing Method

The deep-frozen samples were lyophilised and then suspended in 0.2 mL ammonium acetate 13 Mm pH 7 (0.1% formic acid)/acetonitrile (50:50, *v*/*v*), mixture by vortex, and sonicated for 10 min. Then, the samples were vortexed and centrifuged (10,000× rpm, 5 min, 4 °C). The supernatants were collected and filtered through a 0.22 µm Millex-HV13 membrane (Millipore Corp., Bedford, MA, USA). The faeces and tissue fresh samples were processed as previously described [46]. Briefly, faeces and tissues (kidney, liver and adipose tissue) were prepared by freezing in liquid nitrogen and grinding to a powder with a tissue grinder. Tissue powder (100 mg) was transferred to a microcentrifuge tube and 400 µL of 1% formic acid was added. The sample was vortexed and then sonicated for 3 s. Ice-cold acetonitrile (50 µL) was added to precipitate proteins and the samples were vortexed again, followed by centrifugation at 21,000× *g* for 5 min. The supernatants were removed and kept on ice. This procedure was repeated two times for each tissue and the supernatants were pooled.

2.9.2. Ultra High-Pressure Liquid Chromatography Coupled to Electrospray Ionisation (UHPLC-ESI-QqQ-MS/MS) Analysis

The analysis of the quantitative profile of glucosinolates and their metabolic derivatives in the liver, kidney, adipose tissue, faeces and urine samples was performed by Ultra High-Pressure Liquid Chromatography coupled to Electrospray Ionisation (ESI) and a 6460 tandem Mass Spectrometer with Triple Quadrupole Technology (UHPLC/MS/MS, Agilent Technologies, Waldron, Germany). Chromatographic separation was carried out on a ZORBAX Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm) (Agilent Technologies, Waldron, Germany) through a chromatographic gradient developed by applying different percentages of the solvents A: 13 mM ammonium acetate pH 7 and B: acetonitrile/formic acid (99.99:0.01, *v*/*v*), according to the method previously described [45] (Supplementary Table S1). The identification of the compounds was made through their MS2 fragmentation pattern (mass/charge ratio), applying positive or negative ionisation mode depending on the compound considered at the optimal ESI conditions (Supplementary Table S2), and their retention time in

comparison with authentic standards [45] (Supplementary Table S3). The glucosinolates and isothiocyanates content was expressed as ng/mg tissue fresh weight (fw) on the base of pooled external standard curves freshly prepared each day of analysis.

2.10. Statistics

The concentration data of the compounds present in the biological samples are presented as mean ± standard deviation (SD). For histologic analysis, data are expressed as percentages and the Chi-square test was performed using the SPSS program (Statistical Package for Social Sciences, Chicago, IL, USA) version 17. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Liver, Kidney and Lung Histology

Regarding hepatic lesions, at 1 h, 2 h and 4 h post-ingestion of BLF, no lesions were observed. However, all mice from the CTR (0 h) group (100%) and at 12 h post-ingestion of BLF (100%) presented cell swelling accentuated by hydropic degeneration. One animal at 8 h (33%) and two mice at 24 h (66%) also presented these lesions, with the rest of the animals showing mild hydropic changes, 66% and 33%, respectively. All groups that showed hepatic hydropic changes, namely, CTR (0 h), 8 h, 12 h and 24 h post-ingestion, differed significantly ($p < 0.05$) from the groups without visible liver lesions (1 h, 2 h, and 4 h post-ingestion). Representative images are shown in Figure 1. No lesions were observed in the kidney and lung.

3.2. Quantification of Glucosinolates and Isothiocyanates after BLF Ingestion

Table 1 shows the concentrations of glucosinolates, isothiocyanates and their metabolic derivatives present in the plasma (μmol/L), liver, kidney or faeces (ng/mg tissue fw) in mice at time = 0 h, and following ingestion of 790 mg/kg BLF at 1, 2, 4, 8, 12 and 24 h post-administration. For all time points, samples from three animals were analysed; however, in several analyses the compounds were only detected in one or two animals. The GR, the major glucosinolate present in broccoli leaves [7], was not detected in the plasma, liver, kidney or faeces, and, therefore, is not shown in the table. In turn, its isothiocyanate derivative, SFN, was absorbed into the plasma, peaking at 4 h, and was excreted in the faeces, where it reached the highest concentration at 1 h. The immediate derivative of SFN, SFN-GSH, was not detected in plasma as expected, being only present in the kidney, where it peaked at 1 h, also showing a second peak at 8 h. SFN-GSH was excreted in faeces at high concentrations, peaking at 4 h. Its following derivatives, SFN-CYS and SFN-NAC, were also not detected in plasma, but were found in the liver, peaking at 2 h and 4 h, respectively. In addition, SFN-CYS and SFN-NAC were excreted in faeces at much lower concentrations than SFN-GSH, both showing the highest concentrations at 2 h. GE, the reduced form of GR, was absorbed into the plasma and detected in the liver and kidney, peaking at 4 h. However, its metabolite, erucin, was not detected in the biological samples analysed, and, therefore, is not presented in the table. GB, the second major glucosinolate present in broccoli leaves [7], was detected in plasma and kidney, peaking at 4 h. In turn, I3C, derived from the metabolism of GB, was absorbed into the plasma, and was detected in the liver at high concentrations, peaking also at 4 h. Nevertheless, its derivative, DIM, was not detected in the biological samples analysed, and, therefore, is not shown in the table. GI was also not detected in any of the biological samples analysed; however, its metabolite, iberin, was excreted in the faeces, reaching the highest concentration at 1 h. In the liver, kidney and faeces, none of the compounds analysed were detected at 12 and 24 h, while in the plasma only at 24 h post-ingestion, none of the substances were detected.

Regarding the adipose tissue, none of the compounds analysed were detected and therefore are not presented in the table.

Urine samples were collected and analysed, but in most samples, the volumes were not sufficient to be processed or properly quantified and, therefore, the results are not shown. However, in the urine samples that could be analysed, some compounds were detected, namely, the glucosinolates GR (at 1 h, 2 h, 4 h and 8 h), GB (at 1 h, 2 h and 8 h) and GE (at 1 h, 2 h and 8 h), and the derivatives SFN (at 1 h, 2 h, 4 h and 8 h), SFN-CYS (at 2 h, 4 h and 8 h), SFN-NAC (at 1 h, 2 h, 4 h and 8 h) and I3C (at 1 h, 2 h, 4 h and 8 h).

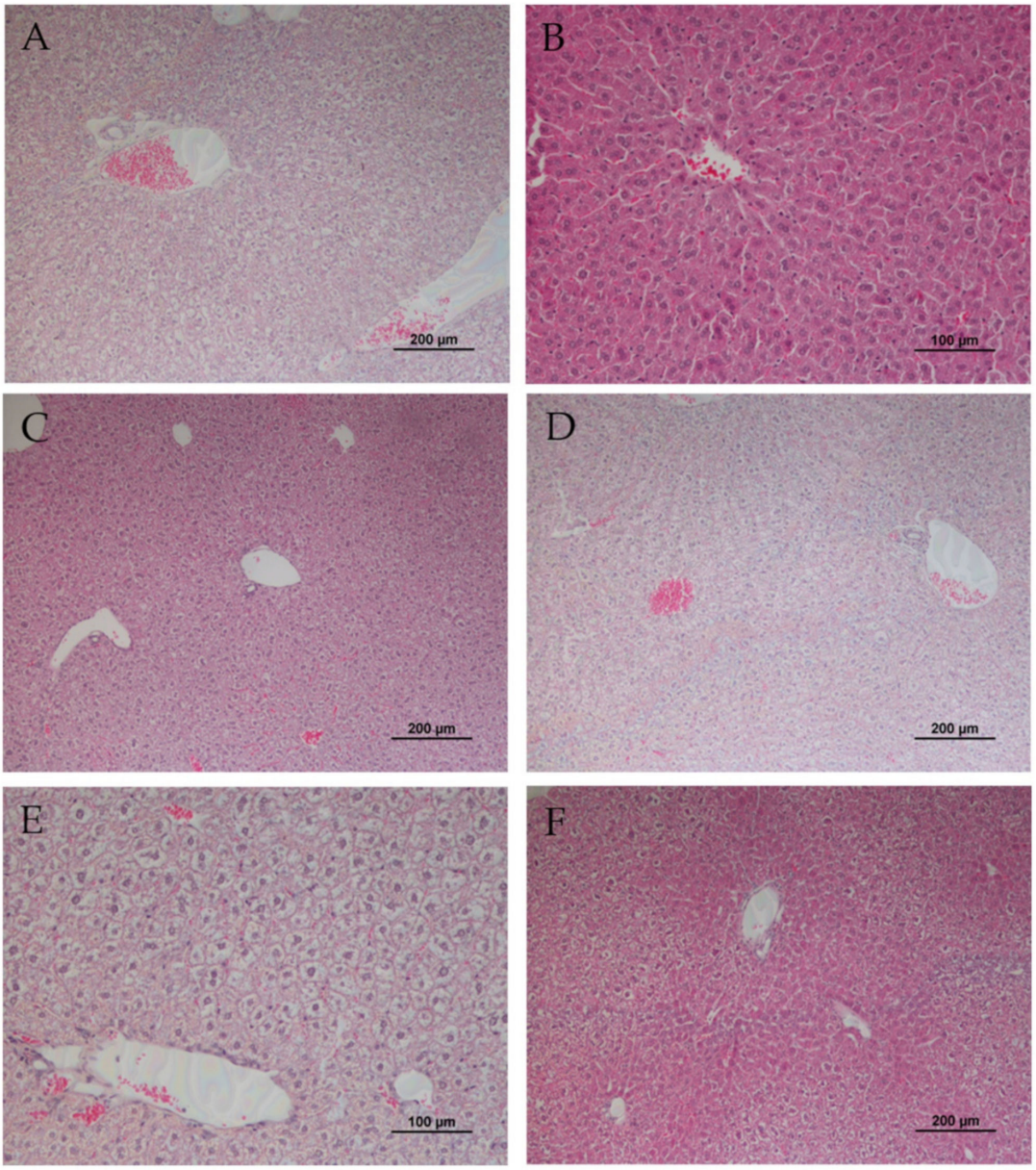

Figure 1. Liver sections from the animals under study (H&E): image (**A**) displays hydropic change with marked cellular swelling visible in an animal of group CTR (0 h); (**B**) normal hepatocytes from an animal of group 1 h post-ingestion of 790 mg/kg broccoli leaf flour (BLF); (**C**) normal liver architecture and hepatocytes from an animal of group 4 h post-ingestion of BLF; (**D**,**E**) hydropic change and cellular swelling in animals from group 12 h post-ingestion of BLF; and (**F**) mild hydropic change, mainly observed on liver zone 2 in an animal from group 24 h post-ingestion of BLF.

Table 1. Concentrations of glucosinolates, isothiocyanates and their metabolic derivatives in the plasma, liver, kidney and faeces after ingestion of 790 mg/kg BLF.

Biological Samples	Time Point	Compounds Detected							
		GE	**GB**	**SFN**	**SFN-GSH**	**SFN-CYS**	**SFN-NAC**	**I3C**	**Iberin**
Plasma (μmol/L)	0 h	–	-	-	-	-	-	-	-
	1 h	-	0.018 ± 0.004 (n = 3)	0.012 ± 0.003 (n = 3)	-	-	-	0.030 ± 0.002 (n = 3)	-
	2 h	0.018 ± 0.002 (n = 3)	0.067 ± 0.012 (n = 3)	0.029 ± 0.003 (n = 3)	-	-	-	0.103 ± 0.007 (n = 3)	-
	4 h	0.031 ± 0.001 (n = 3)	0.094 ± 0.004 (n = 3)	0.040 ± 0.001 (n = 3)	-	-	-	0.145 ± 0.005 (n = 3)	-
	8 h	0.023 ± 0.001 (n = 3)	0.081 ± 0.001 (n = 3)	0.035 ± 0.001 (n = 3)	-	-	-	0.116 ± 0.001 (n = 3)	-
	12 h	-	0.008 ± 0.001 (n = 3)	0.005 ± 0.001 (n = 3)	-	-	-	0.015 ± 0.001 (n = 3)	-
	24 h	-	-	-	-	-	-	-	-
Liver (ng/mg fw)	0 h	-	-	-	-	-	-	-	-
	1 h	-	-	-	-	-	-	4.107 ± 2.083 (n = 2) $	-
	2 h	-	-	-	-	1.195 ± 0.310 (n = 3)	-	4.040 ± 2.522 (n = 3)	-
	4 h	0.112 ± 0.153 (n = 2) $	-	-	-	0.875 ± 0.282 (n = 2) $	0.040 ± 0.001 (n = 2) $	9.584 (n = 1) $	-
	8 h	0.087 ± 0.009 (n = 2) $	-	-	-	0.754 ± 0.208 (n = 3)	0.035 ± 0.001 (n = 2) $	5.017 ± 0.950 (n = 3)	-
	12 h	-	-	-	-	-	-	-	-
	24 h	-	-	-	-	-	-	-	-
Kidney (ng/mg fw)	0 h	-	-	-	-	-	-	-	-
	1 h	-	1.365 ± 0.775 (n = 3)	-	6.841 ± 2.749 (n = 3)	-	-	-	-
	2 h	0.487 ± 0.481 (n = 2) $	1.236 ± 0.262 (n = 3)	-	3.767 ± 0.707 (n = 3)	-	-	-	-
	4 h	0.691 ± 0.100 (n = 2) $	1.796 ± 1.117 (n = 2) $	-	4.525 (n = 1) $	-	-	-	-
	8 h	0.481 ± 0.1227 (n = 3)	1.592 ± 0.568 (n = 3)	-	5.062 ± 1.008 (n = 3)	-	-	-	-
	12 h	-	-	-	-	-	-	-	-
	24 h	-	-	-	-	-	-	-	-

Table 1. *Cont.*

Biological Samples	Time Point	Compounds Detected							
		GE	**GB**	**SFN**	**SFN-GSH**	**SFN-CYS**	**SFN-NAC**	**I3C**	**Iberin**
Faeces (ng/mg fw)	0 h	-	-	-	-	-	-	-	-
	1 h	-	-	0.126 ± 0.142 (*n* = 3)	36.010 ± 9.337 (*n* = 3)	0.239 ± 0.236 (*n* = 3)	0.026 ± 0.020 (*n* = 2)	-	0.235 ± 0.308 (*n* = 3)
	2 h	-	-	0.112 ± 0.059 (*n* = 3)	36.440 ± 8.305 (*n* = 3)	0.402 ± 0.336 (*n* = 3)	0.045 ± 0.010 (*n* = 3)	-	0.037 (*n* = 1) $
	4 h	-	-	0.042 ± 0.016 (*n* = 2) $	37.300 ± 1.663 (*n* = 2) $	0.211 ± 0.113 (*n* = 2) $	0.031 ± 0.013 (*n* = 2) $	-	-
	8 h	-	-	0.025 ± 0.002 (*n* = 3)	7.315 (*n* = 1) $	0.291 ± 0.161 (*n* = 3)	0.027 ± 0.013 (*n* = 3)	-	0.065 ± 0.064 (*n* = 3)
	12 h	-	-	-	-	-	-	-	-
	24 h	-	-	-	-	-	-	-	-

Data are presented as mean ± SD; -, below the limit of quantitation; $, only detected in one or two animals; BLF, broccoli leaf flour; fw, fresh weight; GB, glucobrassicin; GE, glucoerucin; I3C, indole-3-carbinol; SFN, sulforaphane; SFN-GSH, sulforaphane-glutathione; SFN-CYS, sulforaphane-cysteine; SFN-NAC, sulforaphane-N-acetyl-cysteine.

4. Discussion

Drug metabolism is a process of detoxification to reduce the toxicity of xenobiotics and to facilitate their excretion. However, during this process, the resultant metabolites can be biochemically active with therapeutic effects, inactive or toxic [47]. Therefore, it is important to study the formation and bioavailability of metabolites. Broccoli leaves are by-products highly rich in glucosinolates, thus presenting a high potential to be reused for health benefits. Glucosinolates are phytochemicals present in relatively high quantities in *Brassica* vegetables, such as broccoli; however, these compounds are relatively inert and without biological activity [48]. After enzymatic hydrolysis by plant or bacterial myrosinases, biologically active breakdown products are formed, and are responsible for the biological effects observed and attributed to glucosinolates [49]. Intact glucosinolates can be partially absorbed in the stomach; however, due to their hydrophilic nature, these compounds transit to the small intestine where they are degraded by the released active plant myrosinases, which were present in the food product [50]. The remaining intact glucosinolates transit to the colon, where hydrolysis by bacterial myrosinase occurs. The isothiocyanates and other breakdown products are thus essentially absorbed in the small intestine and colon [50]. In the present study, the absorption and excretion of glucosinolates and their breakdown products were quantified in mice after the ingestion of 790 mg/kg BLF. Within 1 h, SFN and I3C become bioavailable in plasma, peaking at 4 h. SFN and its derivatives, SFN-GSH, SFN-CYS and SFN-NAC, were all found in the faeces, with the SFN-GSH being excreted in greater amounts. Of these compounds, only SFN-CYS and SFN-NAC were accumulated in the liver, while SFN-GSH was the only one detected in the kidney. In general, the compounds were detected within 1 h after BLF ingestion, and were detected up to 8 h after administration, except in plasma, where some compounds were detected up to 12 h. In addition, the compounds analysed are not bioaccumulated in adipose tissue. Regarding histopathologic changes, normal tissues were observed 1 h, 2 h and 4 h after BLF ingestion. However, contrary to expectations, hepatic lesions were observed in animals at 0 h, 8 h, 12 h and 24 h post-ingestion of BLF.

4.1. Glucoraphanin (GR), Sulforaphane (SFN) and SFN Conjugates

The glucosinolate GR is the main glucosinolate present in leaves, stems and inflorescences of broccoli [7]. In the present study, the absence of GR in plasma and faeces suggests that this glucosinolate is rapidly and almost entirely hydrolysed after ingestion, although its presence in urine indicates that intact GR may have been partially absorbed. Also, the presence of SFN in the faeces suggests a high hydrolysis of GR by bacterial myrosinase in the colon. SFN is relatively lipophilic, which, combined with a relatively low molecular weight, confers this molecule a high potential for passive diffusion, resulting in rapid absorption into the enterocytes [51,52]. Inside these cells, SFN undergoes extensive first-pass metabolism, where SFN is conjugated with glutathione by GSTs, being then highly effluxed back into the lumen as SFN-GSH conjugate [53]. Luminal non-enzymatic conjugation may also occur as GSH is supplied to the intestinal lumen with the bile [54]. This may explain the high concentration of the SFN-GSH conjugate found in faeces in our study. Moreover, this high conjugation and efflux may translate into a lower bioavailability of the SNF. Nevertheless, non-enzymatic conjugation of isothiocyanates with GSH is reversible and these conjugates can be dissociated [55]. The liver contains high levels of GSH and the highest GST activity; thus, after being absorbed into the portal circulation, the isothiocyanates undergo extensive hepatic first-pass metabolism, similar to what occurs in enterocytes [49]. Nevertheless, in our study, neither SFN nor SFN-GSH were detected in the liver, with only SFN-CYS and SFN-NAC derivatives being detected in this organ. GST P1-1 is the most active isoform on GSH conjugates [56] and is found mainly in extrahepatic tissues, such as the kidney, although GST P isoforms are also present in mouse liver [57]. Moreover, the kidney plays a major role in the conversion of GSH conjugates into the corresponding mercapturic acids, more than the liver [58,59], but in the present work, only SFN-GSH was accumulated in large quantities in the kidney, while SFN-CYS and SFN-NAC derivatives

were only present in urine samples. Regarding plasma, only SFN was detected, but failed to accumulate in the adipose tissue. According to the study by Li and colleagues, an oral administration of 2.5 mg/g body weight of SFN-rich broccoli sprout preparations by female mice resulted in rapid absorption of SFN into the plasma and distribution throughout the lung, heart, kidney, liver, muscle and mammary fat pad [60]. SFN-GSH was also quickly absorbed and distributed throughout the various tissues and at a much higher concentration than SFN, except in mammary fat pad where SFN-GSH was under the limit of detection [60]. The bioavailability of glucosinolate hydrolysis products depends on several factors, such as the initial concentration of glucosinolates and glucosinolate hydrolysis products in the plant material, the hydrolysis due to storage and processing of the plant material, and the nature of the plant material [49,60]. Thus, it is expected that the administration of an SFN-rich broccoli sprout preparation will result in increased bioavailability compared to the administration of a naturally less enriched broccoli leaf flour. SFN is one of the most studied metabolites with promising pharmacological effects [17,61–63]; therefore, a high bioavailability of SFN in the body is desirable. SFN is an indirect antioxidant due to its ability to induce the expression of several antioxidant enzymes via the KEAP1/Nrf2/ARE pathway, which maintain redox potential and activity of free radical scavengers such as vitamins A, C and E [64]. The anticarcinogenic effect of SFN is its most evident and studied therapeutic effect. SFN induces apoptosis and cell-cycle arrest and has antimetastatic effects through several mechanisms of action, such as the induction of the antioxidant enzyme thioredoxin reductase1 (TrxR1), inactivation of inhibitors of apoptosis proteins, activation of MAP kinases and suppression of matrix metalloproteinases, among others [65].

4.2. Glucoerucin (GE) and Erucin

The GE content in broccoli is relatively low [66]. Nevertheless, in the present study, the presence of GE, the precursor of erucin, was detected in the liver, plasma and kidney. The absence of GE in faeces and its bioaccumulation in the kidney indicates that GE is possibly excreted essentially via the urinary tract; in fact, GE was detected in urine samples. Despite the presence of GE in the organism, erucin was not detected in any of the samples, indicating that the hydrolysis of GE was probably very low, being absorbed mainly in its intact form. However, it is possible that in the eventual formation of erucin, it was rapidly converted to SFN through oxidation of the sulphide group. In rats gavaged with erucin, 67% of the applied dose was excreted as SFN-NAC and only 29% as the erucin-NAC conjugate, demonstrating a favourable oxidation of the sulphide group of erucin compared to the reduction of the sulfoxide group in SFN [67]. In mice, pure erucin gavage also revealed its rapid transformation into SFN and subsequent metabolisation via the mercapturic acid pathway [29]. Similar to SFN, erucin is also an important metabolite with therapeutic potential reported in the literature. Erucin has been shown to induce antioxidant enzymes like TrxR1, and to possess hydroperoxide scavenging activity due to its ability to react with H_2O_2 and alkyl hydroperoxides [68,69]. The anticancer effects of erucin have also been shown in in vivo and in vitro models of bladder cancer through downregulation of survivin, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/neu), G(2)/M cell cycle accumulation and apoptosis [70]. Also, erucin demonstrated to have anti-proliferative activity in human lung adenocarcinoma A549 cells through the induction of p53, p21 and PARP-1 cleavage [71]. Recently, erucin was demonstrated to have anticancer effects in an in vitro model of triple-negative breast cancer (TNBC) subtype by inducing apoptosis and autophagy, having also antimetastatic activity [72]. In an in vitro model of renal cancer, erucin induced a concentration-dependent decrease in cell viability and cell-cycle arrest at G2/Mitosis [24].

4.3. Glucobrassicin (GB), Indole-3-carbinol (I3C) and 3,3′-Diindolylmethane (DIM)

The glucosinolate GB was reported to be the second highest glucosinolate present in broccoli leaves (5.23 ± 0.75 mmol/kg dw), occurring in about half the amount of GR (12.23 ± 1.45 mmol/kg dw) [7]. In the present work, GB was found in plasma and ac-

cumulated in the kidney, suggesting that intact GB was partially absorbed. In turn, its hydrolysis product, I3C, was found in plasma and liver. This demonstrates that I3C underwent intestinal absorption after GB breakdown and went to the liver via the portal circulation, then entering the systemic circulation. Under the acidic conditions of the gut, I3C is unstable and is converted into its acidic condensation products, mainly DIM [49,73]. However, in our study, DIM was below the limit of quantitation in all the samples analysed. Possibly, GB was only partially hydrolysed to I3C, which in turn may have resulted in a low generation of DIM, making its quantification difficult. The bioavailability of I3C and DIM is also relevant from a pharmacological point of view. A recent study demonstrated that the administration of I3C attenuated the high salt induced hypertrophy and myocardial stress in rats [21]. In a mouse model of inflammatory bowel disease, the treatment with I3C showed to mitigate chronic colitis through aryl hydrocarbon receptor (AhR)-mediated anti-inflammatory mechanisms [27]. The anti-cancer effects of I3C, as well as DIM, have also been reported, showing antiproliferative and anti-inflammatory activities in several types of cancers, such as breast, colorectal, gastric, liver, oral, pancreatic, and prostate cancer [26]. DIM was also demonstrated to have a therapeutic effect in rheumatoid arthritis by inhibiting proliferation, migration and invasion of rheumatoid arthritis fibroblast-like synoviocytes and reducing pro-inflammatory factors induced by TNF-α in vitro by blocking MAPK and AKT/mTOR pathway, and to prevent inflammation and knee joint destruction in vivo [22].

4.4. Glucoiberin (GI) and Iberin

The glucosinolate GI is also present in broccoli leaves [7]. However, the absence of GI detection in all samples, and the detection of iberin in the faeces, suggests that GI underwent rapid hydrolysis, generating iberin. Nevertheless, this isothiocyanate did not show bioavailability and was excreted in faeces. Iberin also undergoes metabolisation via the mercapturic acid pathway, as after consumption of broccoli, mercapturic acid conjugates of iberin can be detected in human plasma and urine [32]. Iberin is also one of the isothiocyanates with reported therapeutic effects. The treatment with iberin has shown anti-tumour effect against ovarian cancer by inhibiting proliferation and inducing apoptosis through the accumulation of ROS, activation of MAPK signalling, and down-regulation of glutathione peroxidase-1 (GPX1) expression [23]. Recently, the anti-inflammatory effect of iberin was demonstrated in human oral epithelial cells stimulated by TNF-α, in which iberin treatment prevented the activation of specific signal transduction pathways [25]. Also, in a recent study, the treatment with iberin showed protection against renal ischemia-reperfusion injury by significantly reducing the levels of several inflammatory markers [28].

4.5. Glucosinolates and Gut Microbiome

In the absence of plant-derived myrosinase, the gut microbiome plays an important role in the biotransformation of glucosinolates into their respective bioactive isothiocyanates, thus influencing their bioavailability and biological effects [15,74]. On the other hand, the consumption of *Brassica* vegetables greatly influences gut microflora composition [15,74]. Several studies in humans and rodents have been shown that consumption of broccoli can lead to an increase or a decrease in certain species of intestinal bacteria [75–80]. In humans, a *Brassica*-rich diet was shown to decrease sulphate-reducing bacteria [77], which are linked to gastrointestinal diseases [81]. These alterations in gut microbiome induced by *Brassica* vegetables consumption leads to alterations in metabolism, thus influencing host health [15,74]. Thus, it is possible that BLF may exert beneficial health effects through the modulation of the gut microbiome due to SFN and its derivatives being concentrated in the intestine.

5. Conclusions

In conclusion, the absorption of compounds from BLF when administered orally in mice is low. Furthermore, most of the compounds, namely, SFN and its derivatives,

remain in the intestine and are then excreted in the faeces. Nevertheless, the present study shows that SFN and I3C, two isothiocyanates with important biological activity, become bioavailable in plasma after ingestion of 790 mg/kg BLF in mice. As a preliminary study, the present work presents some limitations, namely, it is not possible to know the exact bioavailability of each glucosinolate and isothiocyanate since the exact ingested amount is not possible to determine. In the case of urine, it was not always possible to collect a sufficient amount to be analysed. In this study, we started with the recommended dose of 75 g of vegetables; however, being a pilot study, we used a dose 5 times higher to guarantee the detection of the compounds, since the leaves have a lower content of glucosinolates than the inflorescences or sprouts. Still, in some cases, the amount of the compounds was below the limit of quantitation. Another limitation is the difference between mouse metabolism and human metabolism, which consequently generates differences in the quantity and type of bioavailable compounds, thus making it difficult to interpolate the results obtained in mice to humans. Nevertheless, the application of a dose conversion should be taken into account in future studies to minimise the differences in results between human and mouse metabolism.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals3040039/s1, Table S1: Chromatographic conditions; Table S2: Electrospray Ionisation (ESI) conditions; Table S3: Retention time and MS2 fragmentation pattern (mass/charge (m/z) ratio) of intact glucosinolates and isothiocyanates.

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References

1. Rodriguez-Lopez, A.D.; Melgar, B.; Conidi, C.; Barros, L.; Ferreira, I.C.F.R.; Cassano, A.; Garcia-Castello, E.M. Food Industry By-Products Valorization and New Ingredients: Cases of Study. In *Sustainability of the Food System*; Betoret, N., Betoret, E., Eds.; Academic Press: Cambridge, MA, USA, 2020; pp. 71–99.
2. FAO. Food and Agriculture Organization of the United Nations. FAOSTAT. Available online: https://www.fao.org/faostat/en/#data/QCL (accessed on 10 March 2023).
3. FAO. *Food Wastage Footprint: Impacts on Natural Resources*; FAO: Rome, Italy, 2013.
4. Khedkar, R.; Singh, K. Food Industry Waste: A Panacea or Pollution Hazard? In *Paradigms in Pollution Prevention*; Jindal, T., Ed.; Springer International Publishing: Cham, Switzerland, 2018; pp. 35–47.
5. Parfitt, J.; Barthel, M.; Macnaughton, S. Food Waste Within Food Supply Chains: Quantification and Potential for Change to 2050. *Philos. Trans. R. Soc. B* **2010**, *365*, 3065–3081. [CrossRef] [PubMed]

6. Liu, M.; Zhang, L.; Ser, S.L.; Cumming, J.R.; Ku, K.M. Comparative Phytonutrient Analysis of Broccoli By-Products: The Potentials for Broccoli By-Product Utilization. *Molecules* **2018**, *23*, 900. [CrossRef] [PubMed]
7. Martins, T.; Oliveira, P.A.; Pires, M.J.; Neuparth, M.J.; Lanzarin, G.; Félix, L.; Venâncio, C.; Pinto, M.d.L.; Ferreira, J.; Gaivão, I.; et al. Effect of a Sub-Chronic Oral Exposure of Broccoli (*Brassica oleracea* L. var. *italica*) By-Products Flour on the Physiological Parameters of FVB/N Mice: A Pilot Study. *Foods* **2022**, *11*, 120. [CrossRef] [PubMed]
8. Li, H.; Xia, Y.; Liu, H.-Y.; Guo, H.; He, X.-Q.; Liu, Y.; Wu, D.-T.; Mai, Y.-H.; Li, H.-B.; Zou, L.; et al. Nutritional Values, Beneficial Effects, and Food Applications of Broccoli (*Brassica oleracea* var. *italica* Plenck). *Trends Food Sci. Technol.* **2022**, *119*, 288–308. [CrossRef]
9. Dominguez-Perles, R.; Moreno, D.A.; Carvajal, M.; Garcia-Viguera, C. Composition and Antioxidant Capacity of a Novel Beverage Produced with Green Tea and Minimally-Processed Byproducts of Broccoli. *Innovat. Food Sci. Emerg. Technol.* **2011**, *12*, 361–368. [CrossRef]
10. Drabińska, N.; Ciska, E.; Szmatowicz, B.; Krupa-Kozak, U. Broccoli By-Products Improve the Nutraceutical Potential of Gluten-Free Mini Sponge Cakes. *Food Chem.* **2018**, *267*, 170–177. [CrossRef]
11. Vazquez-Duran, A.; Gallegos-Soto, A.; Bernal-Barragan, H.; Lopez-Perez, M.; Mendez-Albores, A. Physicochemical, Nutritional and Sensory Properties of Deep Fat-Fried Fortified Tortilla Chips with Broccoli (*Brassica oleracea* L. convar. *italica* Plenck). *Flour. J. Food Nutr. Res.* **2014**, *53*, 313–323.
12. Zambelli, R.A.; Pontes, B.C.V.; Pontes, E.R.; Silva, M.L.; Junior, E.C.S.; Pinto, L.I.F.; Melo, C.A.L.; Farias, M.M.; da Costa, C.S.; da Silva, A.C. Broccoli and Carrot Industrial Solid Waste Characterization and Application in the Bread Food Matrix. *Int. J. Nutr. Food Sci.* **2017**, *6*, 9–15. [CrossRef]
13. Fahey, J.W.; Zalcmann, A.T.; Talalay, P. The Chemical Diversity and Distribution of Glucosinolates and Isothiocyanates Among Plants. *Phytochemistry* **2001**, *56*, 5–51. [CrossRef]
14. Prieto, M.A.; López, C.J.; Simal-Gandara, J. Glucosinolates: Molecular Structure, Breakdown, Genetic, Bioavailability, Properties and Healthy and Adverse Effects. In *Advances in Food and Nutrition Research*; Ferreira, I.C.F.R., Barros, L., Eds.; Academic Press: Cambridge, MA, USA, 2019; Volume 90, pp. 305–350.
15. Sikorska-Zimny, K.; Beneduce, L. The Metabolism of Glucosinolates by Gut Microbiota. *Nutrients* **2021**, *13*, 2750. [CrossRef]
16. Maldini, M.; Baima, S.; Morelli, G.; Scaccini, C.; Natella, F. A Liquid Chromatography-Mass Spectrometry Approach to Study "Glucosinoloma" in Broccoli Sprouts. *J. Mass Spectrom.* **2012**, *47*, 1198–1206. [CrossRef] [PubMed]
17. Dinkova-Kostova, A.T.; Kostov, R.V. Glucosinolates and Isothiocyanates in Health and Disease. *Trends Mol. Med.* **2012**, *18*, 337–347. [CrossRef] [PubMed]
18. Martins, T.; Colaco, B.; Venancio, C.; Pires, M.J.; Oliveira, P.A.; Rosa, E.; Antunes, L.M. Potential Effects of Sulforaphane to Fight Obesity. *J. Sci. Food Agric.* **2018**, *98*, 2837–2844. [CrossRef]
19. Mithen, R.; Ho, E. Isothiocyanates for Human Health. *Mol. Nutr. Food Res.* **2018**, *62*, e1870079. [CrossRef] [PubMed]
20. Palliyaguru, D.L.; Yuan, J.M.; Kensler, T.W.; Fahey, J.W. Isothiocyanates: Translating the Power of Plants to People. *Mol. Nutr. Food Res.* **2018**, *62*, e1700965. [CrossRef] [PubMed]
21. Akkiraju, S.; Kanala, S.R.; Khatri, S.; Kutagulla, V.K.; Mallela, V.; Peraman, R. Cardioprotective Potential of Indol-3-Carbinol Against High Salt Induced Myocardial Stress and Hypertrophy in Sprague dawley Rats Besides Molecular Docking on Muscarinic Receptor-2. *Nat. Prod. Res.* **2022**, *36*, 2610–2614. [CrossRef]
22. Du, H.; Zhang, X.; Zeng, Y.; Huang, X.; Chen, H.; Wang, S.; Wu, J.; Li, Q.; Zhu, W.; Li, H.; et al. A Novel Phytochemical, DIM, Inhibits Proliferation, Migration, Invasion and TNF-α Induced Inflammatory Cytokine Production of Synovial Fibroblasts From Rheumatoid Arthritis Patients by Targeting MAPK and AKT/mTOR Signal Pathway. *Front. Immunol.* **2019**, *10*, 1620. [CrossRef]
23. Gong, T.T.; Guo, Q.; Li, X.; Zhang, T.N.; Liu, F.H.; He, X.H.; Lin, B.; Wu, Q.J. Isothiocyanate Iberin Inhibits Cell Proliferation and Induces Cell Apoptosis in the Progression of Ovarian Cancer by Mediating ROS Accumulation and GPX1 Expression. *Biomed. Pharmacother.* **2021**, *142*, 111533. [CrossRef]
24. Guerreiro, Í.; Vidovic, B.; Costa, J.G.; Martins, M.; Ferreira, S.; Oliveira, N.G.; Saraiva, N.; Fernandes, A.S. The Dietary Isothiocyanate Erucin Reduces Kidney Cell Motility by Disturbing Tubulin Polymerization. *Mol. Nutr. Food Res.* **2023**, *67*, e2200581. [CrossRef]
25. Hosokawa, Y.; Hosokawa, I.; Shimoyama, M.; Fujii, A.; Sato, J.; Kadena, K.; Ozaki, K.; Hosaka, K. The Anti-Inflammatory Effects of Iberin on TNF-α-Stimulated Human Oral Epithelial Cells: In Vitro Research. *Biomedicines* **2022**, *10*, 3155. [CrossRef]
26. Reyes-Hernández, O.D.; Figueroa-González, G.; Quintas-Granados, L.I.; Gutiérrez-Ruíz, S.C.; Hernández-Parra, H.; Romero-Montero, A.; Del Prado-Audelo, M.L.; Bernal-Chavez, S.A.; Cortés, H.; Peña-Corona, S.I.; et al. 3,3′-Diindolylmethane and Indole-3-Carbinol: Potential Therapeutic Molecules for Cancer Chemoprevention and Treatment via Regulating Cellular Signaling Pathways. *Cancer Cell Int.* **2023**, *23*, 180. [CrossRef] [PubMed]
27. Riemschneider, S.; Hoffmann, M.; Slanina, U.; Weber, K.; Hauschildt, S.; Lehmann, J. Indol-3-Carbinol and Quercetin Ameliorate Chronic DSS-Induced Colitis in C57BL/6 Mice by AhR-Mediated Anti-Inflammatory Mechanisms. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2262. [CrossRef] [PubMed]
28. Yahiya, Y.I.; Hadi, N.R.; Abu Raghif, A.; Qassam, H.; Al Habooby, N.G.S. Role of Iberin as an Anti-Apoptotic Agent on Renal Ischemia-Reperfusion Injury in Rats. *J. Med. Life* **2023**, *16*, 915–919. [CrossRef] [PubMed]

29. Platz, S.; Piberger, A.L.; Budnowski, J.; Herz, C.; Schreiner, M.; Blaut, M.; Hartwig, A.; Lamy, E.; Hanske, L.; Rohn, S. Bioavailability and Biotransformation of Sulforaphane and Erucin Metabolites in Different Biological Matrices Determined by LC-MS-MS. *Anal. Bioanal. Chem.* **2015**, *407*, 1819–1829. [CrossRef]
30. Zhu, W.; Lerno, L.A.; Cremonini, E.; Oteiza, P.I.; Mastaloudis, A.; Bornhorst, G.M.; Mitchell, A.E. Robust UHPLC-(ESI+)-MS/MS Method for Simultaneous Analysis of Glucoraphanin, Sulforaphane, and Sulforaphane Metabolites in Biological Samples. *ACS Food Sci. Technol.* **2023**, *3*, 1300–1310. [CrossRef]
31. Budnowski, J.; Hanske, L.; Schumacher, F.; Glatt, H.; Platz, S.; Rohn, S.; Blaut, M. Glucosinolates Are Mainly Absorbed Intact in Germfree and Human Microbiota-Associated Mice. *J. Agric. Food Chem.* **2015**, *63*, 8418–8428. [CrossRef]
32. Al Janobi, A.A.; Mithen, R.F.; Gasper, A.V.; Shaw, P.N.; Middleton, R.J.; Ortori, C.A.; Barrett, D.A. Quantitative Measurement of Sulforaphane, Iberin and Their Mercapturic Acid Pathway Metabolites in Human Plasma and Urine Using Liquid Chromatography-Tandem Electrospray Ionisation Mass Spectrometry. *J. Chromatogr. B* **2006**, *844*, 223–234. [CrossRef]
33. Charron, C.S.; Vinyard, B.T.; Ross, S.A.; Seifried, H.E.; Jeffery, E.H.; Novotny, J.A. Absorption and Metabolism of Isothiocyanates Formed from Broccoli Glucosinolates: Effects of BMI and Daily Consumption in a Randomised Clinical Trial. *Br. J. Nutr.* **2018**, *120*, 1370–1379. [CrossRef]
34. Maruthanila, V.L.; Poornima, J.; Mirunalini, S. Attenuation of Carcinogenesis and the Mechanism Underlying by the Influence of Indole-3-Carbinol and Its Metabolite 3,3′-Diindolylmethane: A Therapeutic Marvel. *Adv. Pharmacol. Sci.* **2014**, *2014*, 832161. [CrossRef]
35. Amare, D.E.; Bovee, T.F.H.; Mulder, P.P.J.; Hamers, A.; Hoogenboom, R.L.A.P. Acid Condensation Products of Indole-3-Carbinol and Their *In-Vitro* (Anti)Estrogenic, (Anti)Androgenic and Aryl Hydrocarbon Receptor Activities. *Arab. J. Chem.* **2020**, *13*, 7199–7211. [CrossRef]
36. Orlando, P.; Nartea, A.; Silvestri, S.; Marcheggiani, F.; Cirilli, I.; Dludla, P.V.; Fiorini, R.; Pacetti, D.; Loizzo, M.R.; Lucci, P.; et al. Bioavailability Study of Isothiocyanates and Other Bioactive Compounds of *Brassica oleracea* L. var. *italica Boiled or Steamed: Functional Food or Dietary Supplement? Antioxidants* **2022**, *11*, 209. [CrossRef] [PubMed]
37. Villaño, D.; López-Chillón, M.T.; Zafrilla, P.; Moreno, D.A. Bioavailability of Broccoli Sprouts in Different Human Overweight Populations. *J. Funct. Foods* **2019**, *59*, 337–344. [CrossRef]
38. Nutrition Australia. Australian Dietary Guidelines: Standard Serves. Available online: https://nutritionaustralia.org/fact-sheets/adgs-standard-serves/ (accessed on 10 November 2022).
39. Cox, H.M.; Tough, I.R.; Woolston, A.M.; Zhang, L.; Nguyen, A.D.; Sainsbury, A.; Herzog, H. Peptide YY is Critical for Acylethanolamine Receptor Gpr119-Induced Activation of Gastrointestinal Mucosal Responses. *Cell Metab.* **2010**, *11*, 532–542. [CrossRef] [PubMed]
40. Dhawan, S.S.; Xia, S.; Tait, D.S.; Bundgaard, C.; Bowman, E.; Brown, V.J. Oral Dosing of Rodents Using a Palatable Tablet. *Psychopharmacology* **2018**, *235*, 1527–1532. [CrossRef] [PubMed]
41. Flecknell, P.A.; Roughan, J.V.; Stewart, R. Use of Oral Buprenorphine ("Buprenorphine Jello") for Postoperative Analgesia in Rats—A Clinical Trial. *Lab. Anim.* **1999**, *33*, 169–174. [CrossRef] [PubMed]
42. Zhang, L.; Lee, N.J.; Nguyen, A.D.; Enriquez, R.F.; Riepler, S.J.; Stehrer, B.; Yulyaningsih, E.; Lin, S.; Shi, Y.C.; Baldock, P.A.; et al. Additive Actions of the Cannabinoid and Neuropeptide Y Systems on Adiposity and Lipid Oxidation. *Diabetes Obes. Metab.* **2010**, *12*, 591–603. [CrossRef]
43. Kronenberger, J.P.; Médioni, J. Food Neophobia in Wild and Laboratory Mice (*Mus musculus domesticus*). *Behav. Process.* **1985**, *11*, 53–59. [CrossRef]
44. Martins, T.; Matos, A.F.; Soares, J.; Leite, R.; Pires, M.J.; Ferreira, T.; Medeiros-Fonseca, B.; Rosa, E.; Oliveira, P.A.; Antunes, L.M. Comparison of Gelatin Flavors for Oral Dosing of C57BL/6J and FVB/N Mice. *J. Am. Assoc. Lab. Anim. Sci.* **2022**, *61*, 89–95. [CrossRef]
45. Dominguez-Perles, R.; Medina, S.; Moreno, D.A.; Garcia-Viguera, C.; Ferreres, F.; Gil-Izquierdo, A. A New Ultra-Rapid UHPLC/MS/MS Method for Assessing Glucoraphanin and Sulforaphane Bioavailability in Human Urine. *Food Chem.* **2014**, *143*, 132–138. [CrossRef]
46. Bricker, G.V.; Riedl, K.M.; Ralston, R.A.; Tober, K.L.; Oberyszyn, T.M.; Schwartz, S.J. Isothiocyanate Metabolism, Distribution, and Interconversion in Mice Following Consumption of Thermally Processed Broccoli Sprouts or Purified Sulforaphane. *Mol. Nutr. Food Res.* **2014**, *58*, 1991–2000. [CrossRef]
47. Susa, S.T.; Hussain, A.; Preuss, C.V. Drug Metabolism. In *StatPearls*; StatPearls Publishing: Treasure Island, FL, USA, 2023.
48. Fuentes, F.; Paredes-Gonzalez, X.; Kong, A.N. Dietary Glucosinolates Sulforaphane, Phenethyl Isothiocyanate, Indole-3-Carbinol/3,3′-Diindolylmethane: Anti-Oxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and In Vivo Cancer Chemopreventive Efficacy. *Curr. Pharmacol. Rep.* **2015**, *1*, 179–196. [CrossRef] [PubMed]
49. Holst, B.; Williamson, G. A Critical Review of the Bioavailability of Glucosinolates and Related Compounds. *Nat. Prod. Rep.* **2004**, *21*, 425–447. [CrossRef] [PubMed]
50. Barba, F.J.; Nikmaram, N.; Roohinejad, S.; Khelfa, A.; Zhu, Z.; Koubaa, M. Bioavailability of Glucosinolates and Their Breakdown Products: Impact of Processing. *Front. Nutr.* **2016**, *3*, 24. [CrossRef] [PubMed]
51. Cooper, D.A.; Webb, D.R.; Peters, J.C. Evaluation of the Potential for Olestra to Affect the Availability of Dietary Phytochemicals. *J. Nutr.* **1997**, *127*, 1699S–1709S. [CrossRef] [PubMed]

52. Winiwarter, S.; Bonham, N.M.; Ax, F.; Hallberg, A.; Lennernas, H.; Karlen, A. Correlation of Human Jejunal Permeability (In Vivo) of Drugs with Experimentally and Theoretically Derived Parameters. A Multivariate Data Analysis Approach. *J. Med. Chem.* **1998**, *41*, 4939–4949. [CrossRef]
53. Petri, N.; Tannergren, C.; Holst, B.; Mellon, F.A.; Bao, Y.; Plumb, G.W.; Bacon, J.; O'Leary, K.A.; Kroon, P.A.; Knutson, L.; et al. Absorption/Metabolism of Sulforaphane and Quercetin, and Regulation of Phase II Enzymes, in Human Jejunum In Vivo. *Drug Metab. Dispos.* **2003**, *31*, 805–813. [CrossRef]
54. Samiec, P.S.; Dahm, L.J.; Jones, D.P. Glutathione S-Transferase in Mucus of Rat Small Intestine. *Toxicol. Sci.* **2000**, *54*, 52–59. [CrossRef]
55. Bruggeman, I.M.; Temmink, J.H.M.; van Bladeren, P.J. Glutathione- and Cysteine-Mediated Cytotoxicity of Allyl and Benzyl Isothiocyanate. *Toxicol. Appl. Pharmacol.* **1986**, *83*, 349–359. [CrossRef]
56. Zhang, Y.; Kolm, R.H.; Mannervik, B.; Talalay, P. Reversible Conjugation of Isothiocyanates with Glutathione Catalyzed by Human Glutathione Transferases. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 748–755. [CrossRef]
57. Conklin, D.J.; Haberzettl, P.; Lesgards, J.F.; Prough, R.A.; Srivastava, S.; Bhatnagar, A. Increased Sensitivity of Glutathione S-Transferase P-Null Mice to Cyclophosphamide-Induced Urinary Bladder Toxicity. *J. Pharmacol. Exp. Ther.* **2009**, *331*, 456–469. [CrossRef]
58. Hanna, P.E.; Anders, M.W. The Mercapturic Acid Pathway. *Crit. Rev. Toxicol.* **2019**, *49*, 819–929. [CrossRef] [PubMed]
59. Lock, E.A.; Reed, C.J. Xenobiotic Metabolizing Enzymes of the Kidney. *Toxicol. Pathol.* **1998**, *26*, 18–25. [CrossRef] [PubMed]
60. Li, Y.; Zhang, T.; Li, X.; Zou, P.; Schwartz, S.J.; Sun, D. Kinetics of Sulforaphane in Mice After Consumption of Sulforaphane-Enriched Broccoli Sprout Preparation. *Mol. Nutr. Food Res.* **2013**, *57*, 2128–2136. [CrossRef] [PubMed]
61. Kim, J.K.; Park, S.U. Current Potential Health Benefits of Sulforaphane. *EXCLI J.* **2016**, *15*, 571–577. [CrossRef]
62. Vanduchova, A.; Anzenbacher, P.; Anzenbacherova, E. Isothiocyanate from Broccoli, Sulforaphane, and Its Properties. *J. Med. Food* **2019**, *22*, 121–126. [CrossRef]
63. Yadav, K.; Dhankhar, J.; Kundu, P. Isothiocyanates—A Review of their Health Benefits and Potential Food Applications. *Curr. Res. Nutr. Food Sci.* **2022**, *10*, 476–502. [CrossRef]
64. Boddupalli, S.; Mein, J.R.; Lakkanna, S.; James, D.R. Induction of Phase 2 Antioxidant Enzymes by Broccoli Sulforaphane: Perspectives in Maintaining the Antioxidant Activity of Vitamins A, C, and E. *Front. Genet.* **2012**, *3*, 7. [CrossRef]
65. Gupta, P.; Kim, B.; Kim, S.H.; Srivastava, S.K. Molecular Targets of Isothiocyanates in Cancer: Recent Advances. *Mol. Nutr. Food Res.* **2014**, *58*, 1685–1707. [CrossRef]
66. Mondloch, S.; Scholl, C.D.; Arscott, S.A.; Tanumihardjo, S.A. Glucosinolate and Isothiocyanate Contents of Frozen Broccoli, Brussels Sprouts and a Whole-food Cruciferous Supplement Over Time. *J. Food Nutr. Sci.* **2019**, *1*, 37–48. [CrossRef]
67. Kassahun, K.; Davis, M.; Hu, P.; Martin, B.; Baillie, T. Biotransformation of the Naturally Occurring Isothiocyanate Sulforaphane in the Rat: Identification of Phase I Metabolites and Glutathione Conjugates. *Chem. Res. Toxicol.* **1997**, *10*, 1228–1233. [CrossRef]
68. Melchini, A.; Traka, M.H. Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables. *Toxins* **2010**, *2*, 593–612. [CrossRef] [PubMed]
69. Wang, W.; Wang, S.; Howie, A.F.; Beckett, G.J.; Mithen, R.; Bao, Y. Sulforaphane, Erucin, and Iberin Up-Regulate Thioredoxin Reductase 1 Expression in Human MCF-7 Cells. *J. Agric. Food Chem.* **2005**, *53*, 1417–1421. [CrossRef] [PubMed]
70. Abbaoui, B.; Riedl, K.M.; Ralston, R.A.; Thomas-Ahner, J.M.; Schwartz, S.J.; Clinton, S.K.; Mortazavi, A. Inhibition of Bladder Cancer by Broccoli Isothiocyanates Sulforaphane and Erucin: Characterization, Metabolism, and Interconversion. *Mol. Nutr. Food Res.* **2012**, *56*, 1675–1687. [CrossRef] [PubMed]
71. Melchini, A.; Costa, C.; Traka, M.; Miceli, N.; Mithen, R.; De Pasquale, R.; Trovato, A. Erucin, a New Promising Cancer Chemopreventive Agent from Rocket Salads, Shows Anti-Proliferative Activity on Human Lung Carcinoma A549 Cells. *Food Chem. Toxicol.* **2009**, *47*, 1430–1436. [CrossRef]
72. Bello, I.; Smimmo, M.; d'Emmanuele di Villa Bianca, R.; Bucci, M.; Cirino, G.; Panza, E.; Brancaleone, V. Erucin, an H2S-Releasing Isothiocyanate, Exerts Anticancer Effects in Human Triple-Negative Breast Cancer Cells Triggering Autophagy-Dependent Apoptotic Cell Death. *Int. J. Mol. Sci.* **2023**, *24*, 6764. [CrossRef]
73. Aggarwal, B.B.; Ichikawa, H. Molecular Targets and Anticancer Potential of Indole-3-Carbinol and Its Derivatives. *Cell Cycle* **2005**, *4*, 1201–1215. [CrossRef]
74. Bouranis, J.A.; Beaver, L.M.; Ho, E. Metabolic Fate of Dietary Glucosinolates and Their Metabolites: A Role for the Microbiome. *Front. Nutr.* **2021**, *8*, 748433. [CrossRef]
75. Eve, A.A.; Liu, X.; Wang, Y.; Miller, M.J.; Jeffery, E.H.; Madak-Erdogan, Z. Biomarkers of Broccoli Consumption: Implications for Glutathione Metabolism and Liver Health. *Nutrients* **2020**, *12*, 2514. [CrossRef]
76. Kaczmarek, J.L.; Liu, X.; Charron, C.S.; Novotny, J.A.; Jeffery, E.H.; Seifried, H.E.; Ross, S.A.; Miller, M.J.; Swanson, K.S.; Holscher, H.D. Broccoli Consumption Affects the Human Gastrointestinal Microbiota. *J. Nutr. Biochem.* **2019**, *63*, 27–34. [CrossRef]
77. Kellingray, L.; Tapp, H.S.; Saha, S.; Doleman, J.F.; Narbad, A.; Mithen, R.F. Consumption of a Diet Rich in *Brassica* Vegetables is Associated with a Reduced Abundance of Sulphate-Reducing Bacteria: A Randomised Crossover Study. *Mol. Nutr. Food Res.* **2017**, *61*, 1600992. [CrossRef]
78. Li, F.; Hullar, M.A.; Beresford, S.A.; Lampe, J.W. Variation of Glucoraphanin Metabolism In Vivo and Ex Vivo by Human Gut Bacteria. *Br. J. Nutr.* **2011**, *106*, 408–416. [CrossRef]

79. Wu, Y.; Shen, Y.; Zhu, Y.; Mupunga, J.; Zou, L.; Liu, C.; Liu, S.; Mao, J. Broccoli Ingestion Increases the Glucosinolate Hydrolysis Activity of Microbiota in the Mouse Gut. *Int. J. Nutr. Food Sci.* **2019**, *70*, 585–594. [CrossRef]
80. Zandani, G.; Kaftori-Sandler, N.; Sela, N.; Nyska, A.; Madar, Z. Dietary Broccoli Improves Markers Associated with Glucose and Lipid Metabolism Through Modulation of Gut Microbiota in Mice. *Nutrition* **2021**, *90*, 111240. [CrossRef]
81. Dordević, D.; Jančíková, S.; Vítězová, M.; Kushkevych, I. Hydrogen Sulfide Toxicity in the Gut Environment: Meta-Analysis of Sulfate-Reducing and Lactic Acid Bacteria in Inflammatory Processes. *J. Adv. Res.* **2021**, *27*, 55–69. [CrossRef]