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# Bioaccessible sulforaphane attenuates oxidative stress-driven parainflammation in intestinal epithelial cells

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The dietary consumption of plant-based foods rich in sulforaphane (SFN) and its precursor, glucoraphanin, contributes to health due to SFN's ability to mitigate inflammation. Based on this premise, the present study aims to demonstrate the capacity of bioaccessible breakdown compounds from glucosinolate (SFN) obtained from a broccoli stalk-based ingredient to prevent parainflammatory syndrome associated with oxidative stress, which is strongly linked with a range of metabolic diseases and ageing. UHPLC-ESI-QqQ-MS/MS-based analysis revealed SFN as the only organosulfur compound at a quantifiable concentration in the bioaccessible fraction (4.32 mg per kg dw, corresponding to 0.072  $\mu\text{g mL}^{-1}$  of SFN). The assessment of SFN on the *in vitro* ability to decrease cyclooxygenase-2 (COX-2) concentration and modulate oxidative stress (8-iso-PGF<sub>2 $\alpha$</sub> ) and inflammatory markers (PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>) evidenced a significant anti-inflammatory capacity, which is, to some extent, independent of the weaker capacity to prevent oxidative stress. This result was further confirmed by implementing a model system, which analysed COX-2 expression and the synthesis of isoprostanoids at decreasing concentrations of SFN, starting at the bioaccessible level. The main results obtained emphasise the value of broccoli stalks as a source of antioxidant and anti-inflammatory compounds at operative concentrations in the intestinal lumen and encourage their consideration as a valuable ingredient for health-promoting co-products.

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## Introduction

Inflammation is an adaptive response of living organisms against adverse environmental factors, established to restore the functionality of tissues and organs.<sup>1</sup> Beyond primary inflammation caused by mechanical and chemical insults, microbial infections, and oxidative stress induced by high levels of free radicals resulting from cellular metabolism, chronic inflammatory processes may also occur (Fig. 1).<sup>2</sup> This state is responsible for the transition between basal homeostasis and chronic inflammation, typically triggered by long-term exposure to endogenous or metabolic stress conditions, such as ageing, obesity, or cellular debris, among others. The parainflammatory syndrome serves to maintain tissue function under stress and refers to a low-grade, adaptive immune response that arises between basal homeostasis and chronic inflammation, as a response to persistent endogenous stressors.<sup>1</sup> The continued activation can lead to chronic pathological outcomes that are governed by the activation of several

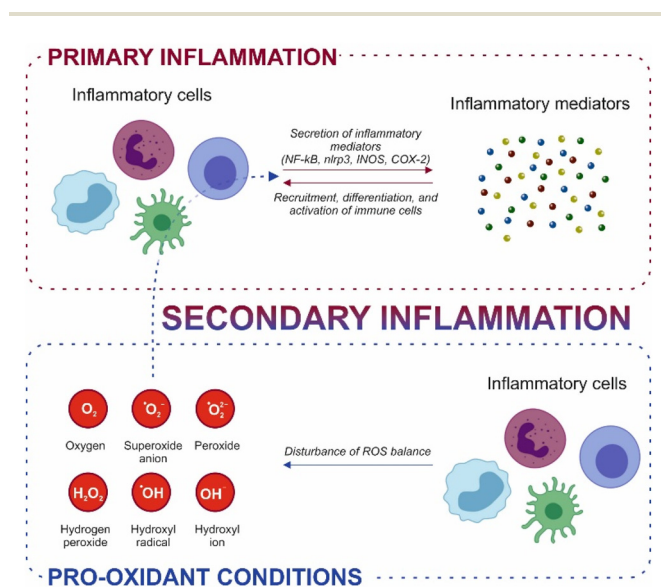


Fig. 1 Scheme of the relationship between free radicals and the activation of the secondary inflammation pathway.

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translocation factors, including nuclear factor  $\kappa\beta$  (NF- $\kappa\beta$ ), inducible nitric oxide synthase (iNOS), or cyclooxygenase-2 (COX-2), among others.<sup>3</sup> These molecules stimulate the recruitment and autoreactivity of immune cells by modifying the interleukin environment, thus perpetuating these pathophysiological conditions.<sup>4</sup>

Among the environmental factors that influence the course of inflammatory processes, diet provides biomolecules that can potentially interact with immune cells' receptors (e.g., fatty acids)<sup>5</sup> or prevent the transcription of proinflammatory mediators (e.g., dietary fibre).<sup>6</sup> Beyond nutrients, other bioactive compounds in plant-based foods, known as phytochemicals, would protect against inflammation.<sup>7,8</sup> In this regard, broccoli (*Brassica oleracea* var. *italica*) has been recognised for its nutritional and phytochemical richness.<sup>9</sup> The bioactive compounds described in broccoli have been demonstrated to be efficient inflammation modulators that support rational medical advice on adjusting dietary patterns. Nonetheless, despite the evidence, the efficiency of broccoli's phytochemicals in preventing the circular transition between oxidative stress and inflammation requires further demonstration (Fig. 1).

When considering the phytochemical compounds present in broccoli, specifically active against inflammation, glucosinolates (GSL) are constituted by a  $\beta$ -D-thioglucose with a sulfonated oxime moiety and a variable side chain (R) derived from the precursor amino acid.<sup>10</sup> The bioactive nature of GSL results from their enzymatic transformation into bioactive derivatives (e.g., isothiocyanates (ITCs)) by hydrolysis catalysed by  $\beta$ -thioglucosidases.<sup>11</sup> Among ITCs, sulforaphane (SFN) is the best-characterised in terms of reactivity with inflammatory mediators. Thus, its capacity to tackle the molecular cascade associated with this process has been broadly demonstrated.<sup>8</sup> As a result, broccoli-based materials have been suggested as functional ingredients against inflammation,<sup>9,12</sup> constituting an opportunity to valorise the derived by-products. Hence, when evaluating current alternatives for these materials beyond enzymatic hydrolysis, it should be noted that bioactive ITC release during gastrointestinal digestion results from the specific physicochemical environment and the enzymatic activity.<sup>7</sup> Therefore, exploring the bioaccessibility of ITCs (the proportion of bioactive compounds released from the food matrix during gastrointestinal digestion and available for absorption, whose stability and release depend on enzymatic, chemical, and matrix interactions that ultimately determine their biological activity<sup>13</sup>) in terms of functionality against parainflammation could provide evidence to reinforce sustainable valorisation alternatives for these materials.

Based on these antecedents, the present work aims to unravel how bioaccessible ITCs, represented by SFN, from broccoli stalks may contribute to lowering the production of mediators of the parainflammatory syndrome (COX-2, and the related isoprostanooids associated with oxidative stress (8-iso-PGF<sub>2 $\alpha$</sub> ) and inflammation (PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>)). The capacity of SFN to prevent parainflammation was further confirmed in a model system by evaluating its dose-dependent efficacy in

modulating COX-2 levels and related oxylipins, thereby highlighting its potential to regulate key pro-inflammatory lipid mediators.

## Results and discussion

### Bioaccessibility of glucosinolates and isothiocyanates from broccoli stalks

The capacity of stabilised broccoli stalk materials to prevent parainflammatory syndrome in the intestinal epithelium was evaluated *in vitro*.<sup>7</sup> According to previous descriptions in the literature by Costa *et al.*, broccoli stalks were pre-processed using minimal processing and optimal dehydration conditions to maximise the concentration of bioaccessible ITCs and indoles in the intestinal lumen after gastrointestinal digestion.<sup>7,14</sup> Applying such processing conditions rendered a broccoli-based material with the following decreasing concentrations of the GSL glucoiberin, glucoraphanin, hydroxy-gluco-brassicin, glucoerucin, glucobrassicin, gluconasturtiin, methoxy-gluco-brassicin, and neo-gluco-brassicin (GI, GR, HGB, GE, GB, PE, MGB, and NGB, respectively): GR (2423.2 mg per kg dw) > GI and MGB (458.9 mg per kg dw, on average) > GE (235.8 mg per kg dw) > GB, HGB, and NGB (106.1 mg per kg dw, on average). As expected, GR, the GSL precursor of the best-characterised ITC (SFN), was the most abundant,<sup>15</sup> explaining the preponderant conditions of SFN among the bioaccessible GSL derivatives. In this sense, the high concentration recorded for GE was also found critical since this GSL is hydrolysed into erucin, which is further converted into SFN,<sup>11</sup> thus contributing extensively to the anti-inflammatory power of the broccoli stalk-based materials.

To confirm the preponderance of SFN in the bioaccessible fraction and thus set up the actual capacity of broccoli stalk powder to prevent parainflammation secondary to oxidative conditions, in the intestinal epithelium, after oral ingestion, the obtained material was exposed to a static *in vitro* simulation of gastrointestinal digestion, which mimics the digestive physiological (mechanical, thermic, chemical, and enzymatic) conditions.<sup>16,17</sup> Quantification of the bioaccessible fraction confirmed that GSL levels in the plant material fell below the limit of detection (<LOD) during gastrointestinal digestion. These findings were in good agreement with previous descriptions on the stability of such sulfur compounds to the physicochemical environment during gastrointestinal digestion, allowing the release of ITCs and indoles into the intestinal lumen.<sup>7,14</sup> Alternatively, on average, among the range of ITCs and indoles monitored (SFN, erucin, iberin, indole-3-carbinol, and 3,4-diindolylmethane (SFN, E, IB, I3C, SFN, and DIM, respectively)), the digestion process rendered 4.32 mg per kg dw (0.072  $\mu\text{g mL}^{-1}$  of the bioaccessible fraction) of SFN, the only GSL-derived product present at quantifiable levels post-digestion. This result is in line with the preponderance of the SFN precursors (GR and GE, oxidised to SFN, especially under acidic conditions<sup>18</sup>) in broccoli stalks,<sup>9,14,19</sup> responsible for giving rise to SFN upon the conversion pathways GR > SFN



and  $GE > E > SFN$ , during gastrointestinal digestion.<sup>14,20,21</sup> Considering the concentration of GR, GE, and SFN in the plant material, the amount of SFN in the bioaccessible fraction appears limited. However, the physicochemical conditions and enzymatic activities during digestion not only convert GSL into ITC and nitrile derivatives but also degrade the newly formed compounds.<sup>22</sup> This fact further supports the stability of SFN against enzymatic degradation during digestion, allowing recovery values ranging from 37% to 64%, much higher than values reported for other ITCs or indoles.<sup>23</sup>

Due to the bioactivities attributed to SFN, its concentration in the intestinal lumen is available for cellular uptake, thus potentially contributing to cutting-edge prevention of molecular pathways implicated in inflammation-associated diseases.<sup>7,24</sup> Based on this evidence, SFN's demonstrated anti-inflammatory capacity provides valuable protection to the intestinal epithelium, thereby attenuating the associated auto-reactive immune response.<sup>7,25</sup> Nonetheless, to our knowledge, the bioactivity of SFN at bioaccessible concentrations remains unproven. In this sense, to evaluate this hypothesis, the current study assessed the capacity of the bioaccessible SFN fraction provided after gastrointestinal digestion of broccoli stalks to prevent parainflammation triggered by oxidative stress. To this end, the digestion products of broccoli stalks were assessed for their capacity to reduce COX-2 expression. This is an inducible cyclooxygenase isoform produced in response to inflammatory and oxidative stimuli,<sup>26</sup> catalysing the synthesis of bioactive lipids, namely prostanoids (PGF<sub>2α</sub> and PGE<sub>2</sub>)<sup>27</sup> and isoprostane (8-iso-PGF<sub>2α</sub>).<sup>23,28</sup> To further demonstrate the anti-inflammatory capacity of SFN, beyond the contribution of additional phytochemicals unavoidably remaining in the bioaccessible fraction, a model system was used to confirm the capacity of SFN alone, at a decreasing range of concentrations starting from those recorded in the bioaccessible fraction of broccoli stalks.

### Modulation of COX-2 by bioaccessible and pure sulforaphane

To shed light on the capacity of bioaccessible SFN to block the molecular mechanisms represented by the synthesis of bioactive lipids involved in parainflammation, changes in the expression of the COX-2 enzyme, responsible for catalysing the first steps of the oxylipin synthesis,<sup>28,29</sup> was monitored. Complementarily, bioactive lipid levels, considered markers of oxidative stress (8-iso-PGF<sub>2α</sub>) and inflammation (PGF<sub>2α</sub> and PGE<sub>2</sub>), were also determined.<sup>27</sup> PGE<sub>2</sub> and PGF<sub>2α</sub> are prototypical COX-2-derived mediators closely associated with the pathophysiology of inflammation and immune system modulation, while 8-iso-PGF<sub>2α</sub>, a non-enzymatic F<sub>2</sub>-isoprostane, is widely recognised as the gold-standard marker of lipid peroxidation and oxidative stress in humans and experimental models. Together, these oxylipins provide integrated results of the components of the parainflammatory response targeted by bioaccessible SFN.<sup>30–32</sup>

With this objective, an *in vitro* model of oxidative stress in the intestinal epithelium (Caco-2 cells) was chosen for these determinations based on the activity of the bioaccessible frac-

tion of bioactive compounds ingested by diet or nutraceuticals.

The *in vitro* monolayer of ciliated intestinal epithelium was exposed to a prooxidant stimulus (50 μM H<sub>2</sub>O<sub>2</sub>) to induce the parainflammatory phenotype and the associated signalling pathways, a central event being the upregulation of the COX-2 expression.<sup>33</sup> The level of COX-2 was monitored for 1 hour under prooxidant conditions, which provided the highest enzyme expression.<sup>27,34</sup>

Intestinal epithelial cells exposed to the prooxidant environment in the absence of bioactive compounds (positive control) significantly augmented the level of COX-2 (up to 147.56 ng mL<sup>-1</sup>, on average, almost 2.5-fold higher than the concentration recorded in basal cells untreated with H<sub>2</sub>O<sub>2</sub> – 56.86 ng mL<sup>-1</sup>) (Fig. 2).

Alternatively, when epithelial cells were pretreated with the bioaccessible fraction of broccoli stalks containing SFN at 0.072 μg mL<sup>-1</sup> (diluted 1:10, v/v, to achieve the final concentration of 0.007 μg mL<sup>-1</sup> in the well), the increase of COX-2 expression was prevented, resulting in an average value of 92.07 ng mL<sup>-1</sup>, significantly lower ( $p < 0.001$ ) than the positive control (black dotted line, Fig. 2).

These results suggest that the bioaccessible SFN can downregulate COX-2 expression induced by prooxidant conditions (50 μM H<sub>2</sub>O<sub>2</sub>). It is important to note that this effect reflects modulation of COX-2 expression rather than direct inhibition of COX-2 enzymatic activity. Thus, SFN is not a classical active-site inhibitor of COX-2; instead, its anti-inflammatory activity is primarily mediated through upstream regulatory mechanisms, including suppression of NF-κB and MAPK signalling and activation of the Nrf2/HO-1 pathway, which collectively regulate COX-2 gene transcription.<sup>35,36</sup> As a consequence, relatively low concentrations may elicit marked biological effects through signal-amplification mechanisms at the transcriptional level, without requiring direct interaction with the enzyme catalytic site. Nonetheless, despite the significant correlation

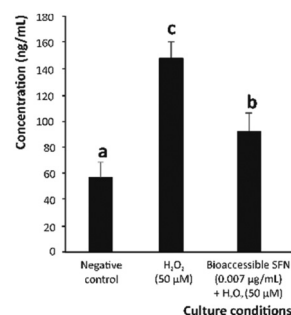


Fig. 2 Modulation of the cyclooxygenase-2 (COX-2) expression (ng mL<sup>-1</sup>) recorded at 1 hour after the application of the oxidative stimulus in the absence and presence of digestion products (bioaccessible SFN (diluted 1:10 (v/v) to achieve the final concentration of 0.007 μg mL<sup>-1</sup> in the wells) from broccoli stalks). Distinct lowercase letters indicate values significantly different at  $p < 0.001$  according to one-way analyses of variance (ANOVA) and Tukey's multiple range test ( $n = 3$ ).



demonstrated for bioaccessible SFN concerning the anti-inflammatory capability of broccoli stalks,<sup>7</sup> the gastrointestinal digestion of broccoli stalk powder might render additional bioactive compounds, even at concentrations lower than the limit of quantification (LOQ) (in trace amounts, *e.g.*, 5-caffeoylquinic and *p*-coumaroylquinic acids<sup>14</sup>).

These additional bioactive molecules could contribute to COX-2 expression inhibition and thus enhance the anti-inflammatory potential of broccoli stalk bioactive components, or they could antagonise SFN's biological activity, thereby limiting its actual anti-inflammatory capacity. To overcome this constraint and further confirm the potential of bioaccessible SFN in reducing COX-2 expression, a model system consisting of SFN solutions at a range of decreasing amounts starting from almost the concentration recorded in the bioaccessible fraction (0.100, 0.050, 0.025, 0.013, and 0.007  $\mu\text{g}$  SFN per mL) was developed and assessed (Fig. 3).

When evaluating the capacity of decreasing SFN concentrations (diluted 1:10 (v/v) in culture media to achieve the final concentrations of 0.0100, 0.0050, 0.0025, 0.0013, and 0.0007  $\mu\text{g}$  SFN per mL in the wells) free of additional bioactive compounds, it was found that all of them reduced the expression of COX-2 at matching (not significantly different) levels (by 53.6% relative to cells maintained under prooxidant conditions (positive control: black dotted line in Fig. 3)), which differed from the inhibitory capacity exerted by bioaccessible SFN, which lessened the COX-2 expression recorded in cells exposed to an oxidative environment by 37.6% (Fig. 3).

This result reinforces previous evidence of SFN's key role in preventing parainflammatory syndrome through consumption

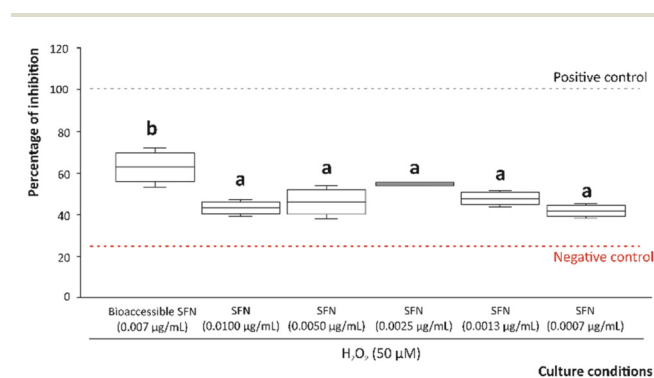
of broccoli stalk-based products, which provide a bioactive bioaccessible fraction capable of downregulating COX-2 expression. Interestingly, beyond the inhibitory capacity of isolated SFN at concentrations matching those recorded in the bioaccessible fraction, the inhibitory efficiency was maintained up to the lowest concentration tested (0.0007  $\mu\text{g}$  SFN per mL) that significantly reduced the expression of COX-2 induced by the prooxidant environment; however, this did not allow recovery of the basal level (negative control, consisting of Caco-2 cells exposed to digestion-blank supplemented culture media in the absence of SFN and  $\text{H}_2\text{O}_2$ , thus providing the basal COX-2 expression level) (red dotted line in Fig. 3), indicating that the inhibitory effect was specifically associated with SFN exposure. This result does not rule out the contribution of additional bioaccessible molecules from broccoli stalks that could exert complementary bioactivities, thereby enhancing the overall anti-inflammatory effect. Indeed, to date, a knowledge gap persists regarding the identification of molecules with bioactive potential that enable functional interactions (synergistic or antagonist) modulating anti-inflammatory responses,<sup>37</sup> particularly among compounds with diverse chemical characteristics.

Concerning the mechanisms responsible for SFN inhibition of COX-2-dependent inflammation, this compound inhibits NF- $\kappa\text{B}$  and MAPK pathways.<sup>38</sup> These mechanisms reduce additional inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8).<sup>39</sup> To characterise bioaccessible SFN's effects on parainflammation, we specifically targeted the COX-2 oxylipin axis (PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , 8-iso-PGF<sub>2 $\alpha$</sub> ), as these are established functional markers of this cascade.<sup>40,41</sup> Hence, while monitoring additional protein-level markers such as malondialdehyde (MDA), glutathione (GSH), or apoptosis endpoints would provide complementary profiling,<sup>30</sup> our targeted panel offers directed evidence of COX-2-dependent parainflammation modulated by bioaccessible SFN.

The observed inhibition of COX-2 and oxylipin production by bioaccessible SFN aligns with its established mechanisms involving NF- $\kappa\text{B}$  and MAPK pathway suppression<sup>38</sup> and potent activation of the Nrf2/Keap1 antioxidant pathway, inducing HO-1.<sup>42,43</sup> Although direct assessment of these upstream regulators was beyond our experimental scope (specifically targeting the functional COX-2 oxylipin axis in a parainflammation model), these mechanisms likely contribute to SFN's modulation of isoprostanes and prostanoids observed using bioaccessible broccoli stalk-derived SFN.

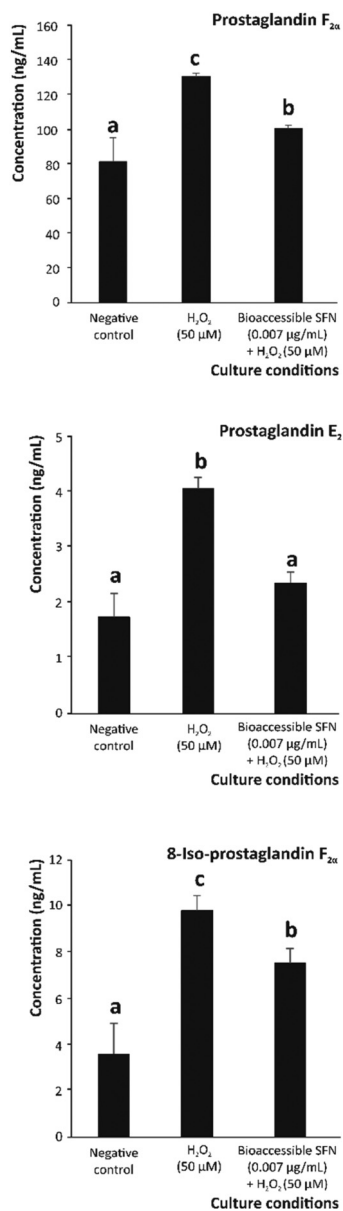
### Effect of sulforaphane on the expression of oxidative stress and inflammation-related oxylipins

To explore the capacity of bioaccessible SFN to modulate the production of isoprostanooids, and specifically the considered gold standard for monitoring oxidative stress (8-iso-PGF<sub>2 $\alpha$</sub> ), the intestinal epithelium was exposed to a pro-oxidant environment (50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ). This treatment augmented the 8-iso-PGF<sub>2 $\alpha$</sub>  concentration from 3.6 ng mL<sup>-1</sup> in unstimulated controls to 9.8 ng mL<sup>-1</sup>, on average (a 1.7-fold increase) after 1 hour (Fig. 4).



**Fig. 3** Percentage reduction in cyclooxygenase-2 (COX-2) concentration by bioaccessible SFN (diluted (v/v) 1:10 in culture media to achieve the final concentration of 0.007  $\mu\text{g}$  mL<sup>-1</sup> in the well) recorded at 1 h after oxidative stimulus (50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) in the absence and presence of digestion products, and in a model system using dilutions (0.0100, 0.0050, 0.0025, 0.0013, and 0.0007  $\mu\text{g}$  mL<sup>-1</sup>, final concentrations in the well) of the authentic SFN standard. Positive control (black dotted line): COX-2 expression in intestinal epithelial cells exposed to prooxidant conditions in the absence of bioactive compounds. Negative control (red dotted line): COX-2 expression in intestinal epithelial cells under basal (non-prooxidant) conditions. Distinct lowercase letters indicate values significantly different at  $p < 0.001$  according to one-way analyses of variance (ANOVA) and Tukey's multiple range test ( $n = 3$ ).





**Fig. 4** Modulation of the prostanoide concentration ( $\text{ng mL}^{-1}$ ) (8-iso-PGF<sub>2 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>) by bioaccessible SFN (diluted (v/v) 1:10 in culture media to achieve the final concentration of  $0.007 \mu\text{g mL}^{-1}$  in the well) at 1 hour post-oxidative stimulus ( $50 \mu\text{M H}_2\text{O}_2$ ). Distinct lowercase letters indicate values significantly different at  $p < 0.001$  according to one-way analyses of variance (ANOVA) and Tukey's multiple range test ( $n = 3$ ).

Alternatively, pretreating cells with the bioaccessible fraction of broccoli stalk containing  $0.072 \mu\text{g SFN per mL}$  (diluted 1:10, v/v, to achieve the final concentration of  $0.007 \mu\text{g mL}^{-1}$  in the well), mitigated the increase in isoprostanes, resulting in a 23.5% lower 8-iso-PGF<sub>2 $\alpha$</sub>  concentration relative to the positive control (black dotted line) (Fig. 4).

The secondary inflammatory response resulting from the oxidative status<sup>2</sup> was monitored by measuring prostanoide levels of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>. These assessments demonstrated

that, when exposed to oxidative conditions for 1 hour (positive control, black dotted line), Caco-2 cells produced up to  $129.8 \text{ ng of PGF}_{2\alpha}$  per mL (37.8% higher than untreated cells, on average). These growth conditions also increased the PGE<sub>2</sub> concentration by 67.2% (from  $1.7$  to  $4.1 \text{ ng mL}^{-1}$ ) (Fig. 4).

Consistent with our initial hypothesis, pretreatment of Caco-2 cells with bioaccessible SFN prevented the increase in both PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> levels, resulting in 23.0% and 42.0% lower concentrations, respectively, in comparison with the positive control (black dotted line), although these treatments did not allowed achieving the basal conditions (negative control, red dotted line) (Fig. 4).

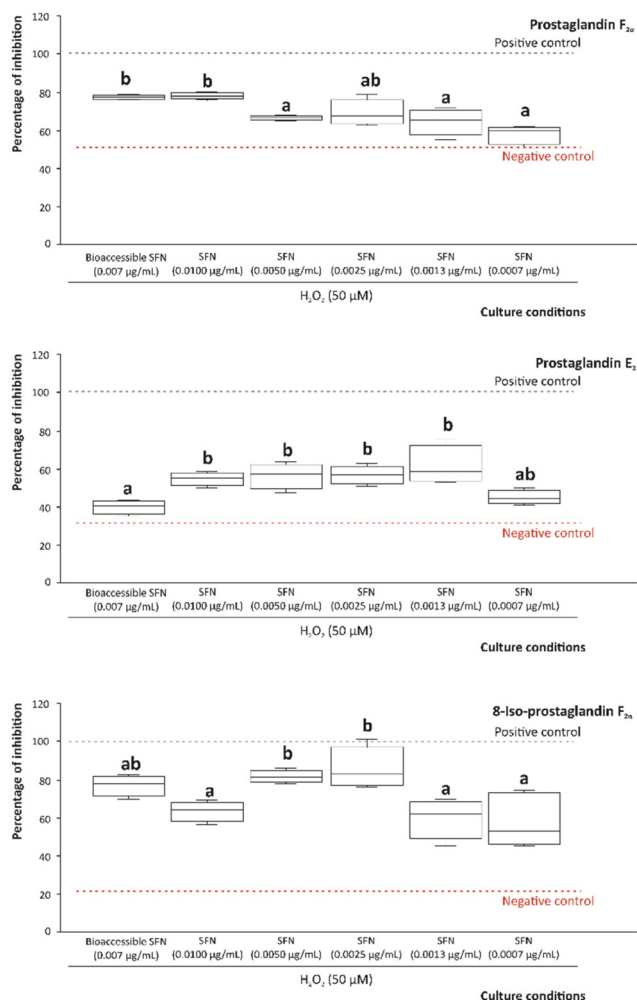
When analysing the capacity of bioaccessible SFN to modulate the expression of COX-2, further confirmation of its potential to inhibit the secretion of prostanoids (markers and mediators of oxidative stress and inflammation) was obtained using a model system (Fig. 5). This approach shed light on the capacity of isolated SFN (free of additional phytochemicals) to adjust the secretion of 8-iso-PGF<sub>2 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> , and PGE<sub>2</sub>.

For 8-iso-PGF<sub>2 $\alpha$</sub> , a somewhat fluctuating pattern was observed. Although bioaccessible SFN showed a trend toward reducing this oxidative stress marker by 29.4%, on average, compared to the positive control (black dotted line), the reduction was not significant across all concentrations (Fig. 5). The limited capacity aligns with the structural constraints of SFN, which confer indirect antioxidant properties to this ITC.<sup>44</sup> This type of antioxidant compound activates the Keap1/Nrf2/ARE pathway, thereby inducing the expression of cytoprotective enzymes that catalyse the synthesis of direct antioxidants. Therefore, the indirect antioxidant function of SFN is delayed<sup>45</sup> and could not be achieved, which is consistent with its limited ability to control the cellular response to oxidative stress *via* isoprostanoid upregulation. In contrast, SFN's well-established ability to modulate inflammatory mediators<sup>46</sup> is evidenced by reducing the concentration of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> across nearly all tested concentrations. Interestingly, although both prostanoids decreased, their specific responses differed.

Upon the model system, the lowering concentrations of the authentic standard of SFN gradually reduced the secretion of PGF<sub>2 $\alpha$</sub>  from the level achieved by bioaccessible SFN ( $0.007 \mu\text{g mL}^{-1}$ ) up to  $74.8 \text{ ng mL}^{-1}$ , corresponding to PGF<sub>2 $\alpha$</sub>  reductions of 22.9% and 42.4%, respectively (Fig. 5). Alternatively, the level of PGE<sub>2</sub> achieved upon the simulation was significantly higher in almost the entire range of SFN concentration tested (except for  $0.0007 \mu\text{g}$  of SFN per mL) (Fig. 5).

This result suggests that bioaccessible SFN exhibits a robust anti-inflammatory capacity by modulating specific mediators, independently of the origin of the process (direct inflammation or parainflammation). However, this biological power could be, to some extent, dependent on the presence of additional bioactive compounds in the media that would fine-tune the anti-inflammatory ability of individual organosulfur compounds.<sup>47</sup> Indeed, these additive or synergic effects could be attributed, for instance, to caffeoylquinic and *p*-coumaroylquinic acids, also described in the bioaccessible fraction of





**Fig. 5** Modulation of the concentration ( $\text{ng mL}^{-1}$ ) of the isoprostane 8-iso-PGF<sub>2 $\alpha$</sub>  and the prostaglandins PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> by bioaccessible SFN ( $0.007 \mu\text{g mL}^{-1}$ , final concentration in the well) recorded at 1 h after oxidative stimulus ( $50 \mu\text{M H}_2\text{O}_2$ ) in the absence and presence of digestion products, and in a model system using dilutions (0.0100, 0.0050, 0.0025, 0.0013, and  $0.0007 \mu\text{g mL}^{-1}$ , final concentrations in the well) of the authentic SFN standard. Positive control (black dotted line): prostanoid concentrations in wells with intestinal epithelial cells exposed to prooxidant conditions in the absence of bioactive compounds. Negative control (red dotted line): prostanoid concentrations in wells with intestinal epithelial cells under basal (non-prooxidant) conditions. Distinct lowercase letters indicate values significantly different at  $p < 0.001$  according to one-way analyses of variance (ANOVA) and Tukey's multiple range test ( $n = 3$ ).

broccoli stalks,<sup>14</sup> on which anti-inflammatory capacity has also been demonstrated.<sup>48</sup>

According to the main results described in the present work, the modulatory capacity of SFN on the secretion of PGs *via* COX-2 can influence the onset and progression of the para-inflammatory syndrome, especially given the close connection reported between the inflammatory PG profile and the level of interleukins.<sup>49</sup>

## Experimental

### Chemicals and reagents

The standards of GSL (GI, GR, HGB, GE, GB, PE, MGB, and NGB) and ITCs and indoles (SFN, E, IB, I3C, SFN, and DIM) were obtained from PhytoPlan GmbH (Heidelberg, Germany). The  $\beta$ -glucuronidase from *Helix pomatia* (type H-2), the digestive enzymes porcine pepsin (P6887) and pancreatin (P7545,  $8 \times \text{USP}$ ), and pancreatic lipase from porcine pancreas (L3126) were obtained from Sigma-Aldrich (St Louis, MO, USA). Standards of the isoprostanooids 8-iso-PGF<sub>2 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> , and PGE<sub>2</sub> were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The COX-2 quantification kit was obtained from Abcam (ab267646) (Cambridge, UK). Solid phase extraction (SPE) cartridges (Strata X-AW) were provided by Phenomenex (Torrance, California, USA), and all LC-MS solvents were obtained from J. T. Baker (Phillipsburg, NJ, USA). Ultrapure water was produced using a Millipore water purification system (Bedford, MA, USA). Trypsin-ethylenediaminetetraacetic acid (EDTA), Eagle's Minimum Essential Medium (EMEM), L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin, and essential amino acids were obtained from Gibco (Thermo Fisher Scientific, Madrid, Spain), and the 24-well plates from Corning (New York, USA).

### Plant material

Commercial seeds of the broccoli plants (*Brassica oleracea* var. *italica*, cultivar 'Parthenon') were obtained from Cytoplasmic Male Sterility provided by SAKATA Seed Ibérica (Alicante, Spain). Broccoli plants were cultivated in the fall-winter cycle of 2024, in the Experimental Farm of CEBAS-CSIC, "La Matanza" (Santomera, Murcia, SE Spain;  $38^{\circ}6'14'' \text{ N}$ ,  $1^{\circ}1'59'' \text{ W}$ ). Harvesting was performed when plants presented mature commercial flowering heads with quality parameters corresponding to the "marketable" class, and inflorescences were manually separated from the stalks. The period between sampling and processing was less than 4 hours to avoid degradation. Once in the laboratory, broccoli stalks were processed according to the conditions previously described to obtain a stabilised material providing high concentrations of bioaccessible ITC.<sup>7,14</sup> The dehydrated samples were ground into a fine powder, stored, and protected from light until the extraction of GSL and ITC according to previously described methodologies<sup>50</sup> and simulated *in vitro* gastrointestinal digestion.

### *In vitro* simulated gastrointestinal digestion

Gastrointestinal digestions were performed on dehydrated broccoli stalk powder (500 mg) following the methodology described in the literature,<sup>16,17</sup> with minor modifications.<sup>20,51</sup> The gastrointestinal digestions were carried out in the presence of pepsin, pancreatic, and pancreatic lipase as digestive enzymes. After digestion, the samples were centrifuged at  $1600g$  for 5 min at  $4 \text{ }^{\circ}\text{C}$  to separate the bioaccessible fraction (30 mL of the supernatant) from the residual material (pellet). The former was filtered through a  $0.22 \mu\text{m}$  polyvinylidene flu-



oride (PVDF) filter (Millipore, MA, USA) and frozen at  $-80\text{ }^{\circ}\text{C}$  until UHPLC-QqQ-MS/MS analysis.

#### Assessment of the quantitative glucosinolate profile by LC-MS

The determination of the GSL content in broccoli stalks was performed based on a previously published methodology.<sup>50</sup> The concentration of the identified compounds was calculated using standard curves freshly prepared on each analysis day and expressed as mg per kg dw.

#### UHPLC-ESI-QqQ-MS/MS analysis of bioaccessible sulforaphane

The assessment of the broccoli stalk powder and its bioaccessible fraction on the ITC and nitrile content was developed according to the processing and analysis methodology described previously.<sup>20,21,52</sup> Briefly, the GSL, ITCs, and nitriles in the filtered bioaccessible fractions were resolved chromatographically and identified by UHPLC/MS/MS (a UHPLC system coupled with a 6460 tandem mass spectrometer with triple quadrupole technology (UHPLC/MS/MS, Agilent Technologies, Waldbronn, Germany)). Chromatographic separation was carried out on a ZORBAX Eclipse Plus C-18 Rapid Resolution HD ( $2.1 \times 50\text{ mm}$ ,  $1.8\text{ }\mu\text{m}$ ) (Agilent Technologies). The column temperature was held at  $10\text{ }^{\circ}\text{C}$  (left and right). Dynamic multiple reaction monitoring (MRM) was performed in positive ion mode and preferential transitions of the corresponding analytes. Dwell time was 30 ms for all MRM transitions. The mobile phases employed were solvent A: ammonium acetate, 13 mM (pH 4 with acetic acid), and solvent B: acetonitrile/acetic acid (99.9:0.1, v/v). The flow rate was  $0.3\text{ mL min}^{-1}$  using the linear gradient scheme ( $t$  in min; % B): (0.00; 12), (0.20; 20), (1.00; 52), (2.50; 95), and (2.51; 12), and a post-run of 1.5 min for the column equilibration. The optimal ESI conditions for maximal detection of the analytes were: gas temperature,  $225\text{ }^{\circ}\text{C}$ ; sheath gas temperature,  $350\text{ }^{\circ}\text{C}$ ; capillary voltage, 3500 V; nozzle voltage, 1250 V; sheath gas flow, 12%; gas flow, 10; nebuliser, 40. The MS parameter fragmentor (ion optics capillary exit voltage) and collision energy for each target analyte to generate the most-abundant product ions for the MRM mode were set up according to the original method.<sup>21</sup> Data acquisition was performed using MassHunter software version B.08.00 (Agilent). The concentration of SFN was calculated using standard curves freshly prepared on each analysis day and expressed as mg per kg dw ( $\mu\text{g mL}^{-1}$ ). The LOD and LOQ for sulforaphane were  $0.0012\text{ }\mu\text{g mL}^{-1}$  and  $0.0066\text{ }\mu\text{g mL}^{-1}$ , respectively.

#### Cell line, culture, and experimental conditions

The human colon adenocarcinoma (Caco-2, TCCHTB37) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) (passage number between 15 and 20). Caco-2 cells were seeded at a density of  $6 \times 10^5$  cells per well on 24-well plates (Costar, Corning, Birmingham, UK). Cells were grown in EMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, and 1% non-essential amino acids, at  $37\text{ }^{\circ}\text{C}$  under a humidified atmosphere containing 5%  $\text{CO}_2$ .

When confluent, the Caco-2 cells were allowed to differentiate into pseudo-ciliated intestinal epithelial cells for 21 days before the experiments, replacing the culture media every 48–72 hours. Subsequently, the culture medium was replaced with an FBS-free medium with bioaccessible SFN. With this objective, the bioaccessible fraction of broccoli stalks obtained by the *in vitro* simulated gastrointestinal digestion was diluted (1:10, v/v) in the culture media consisting of EMEM supplemented only with 2 mM glutamine and 1% non-essential amino acids, thus obtaining the SFN solution at  $0.007\text{ }\mu\text{g}$  of SFN per mL (final concentration in the well) used to pre-treat Caco-2 cells.

In addition, decreasing concentrations of authentic SFN standard (containing the bioaccessible level; 0.100, 0.050, 0.025, 0.013, and  $0.007\text{ }\mu\text{g}$  SFN per mL) were used to supplement the Caco-2 culture media following an equal procedure described for the bioaccessible fraction (1:10, v/v), thus obtaining the final concentrations in the well of 0.0100, 0.0050, 0.0025, 0.0013, and  $0.0007\text{ }\mu\text{g}$  SFN per mL. The control cells were exposed to culture media supplemented with an equal volume of the diluted blank of the digestion. After 24 h of exposure, OS was induced by treating cells with  $50\text{ }\mu\text{M H}_2\text{O}_2$  (final concentration) in the presence and absence of bioactive compounds, with the latter considered the positive control. Both the supernatant and detached cells were collected after 1 h of exposure to the pro-oxidant environment in the presence of bioaccessible SFN and SFN at different concentrations, to assess the ability of both to modulate the concentration of COX-2 and derived oxylipins, respectively.

#### COX-2 assessment by ELISA

The supernatants obtained from the Caco-2 culture following the specified treatments were assessed for COX-2 expression using specific Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the manufacturer's instructions (ab267646 Abcam, Cambridge, UK).

#### Extraction and UHPLC-ESI-QqQ-MS/MS analysis of cells' oxylipins

The assessment of supernatants on the isoprostanoid content was developed according to the methodology described previously.<sup>53</sup> Growth media were cleaned up by SPE using the Strata X-AW cartridges, according to the procedure described by Medina *et al.*<sup>54</sup> The eluted compounds were dried in a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA) and, afterwards, reconstituted with deionised Milli-Q/0.1% formic acid in ACN (90:10, v/v) and filtered through a  $0.22\text{ }\mu\text{m}$  PVDF filter (Millipore, MA, USA). Subsequently, the concentrations of 8-iso-PGF<sub>2 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> , and PGE<sub>2</sub> were analysed using a UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), according to the methodology and settings described in the literature.<sup>54</sup> The isoprostanoids were monitored by multiple transitions (precursor ions to product ions), and retention times, in comparison with authentic standards. The concentration was set up according to standard curves prepared each analysis day. Data acquisition and pro-



cessing were performed using the MassHunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany).

### Statistical analysis

All experimental conditions were performed in triplicate ( $n = 3$ ), and the data were expressed as the mean  $\pm$  standard deviation (SD). According to the normal distribution and homogeneity of variance of the data (determined by the Shapiro–Wilk (<50 samples) and Levene tests, respectively), the obtained results were subjected to one-way analyses of variance (ANOVA). When statistical differences were identified, the variables were compared using Tukey's multiple range test. Significant differences were set at  $p < 0.05$ . Data were processed using the SPSS 29.0 software package (LEAD Technologies, Inc., Chicago, IL, USA).

## Conclusions

The results retrieved from the present study further support the potential of broccoli stalks, an underexploited by-product, as a source of SFN by describing the bioaccessibility (digestive release and stability). Most ITCs and indoles expected from the GSL profile of the plant material were not present, at quantifiable concentrations, in the bioaccessible fraction due to their instability under the physicochemical conditions and enzymatic activity during digestion. Despite the degradation of a range of ITCs and indoles, when evaluating the capacity to prevent the parainflammatory syndrome secondary to oxidative stress, it was found that bioaccessible SFN prevents parainflammation. The assessment of the biological activity of bioaccessible SFN using a model system mimicking its concentrations in the intestinal lumen suggested that this organosulfur compound, with putative bioactivity, is responsible for a primary anti-inflammatory activity and, to a lesser extent, for preventing oxidative stress. Together, these results would suggest combining broccoli stalk powder with additional ingredients obtained from agro-food by-products or edible material sources of bioactive compounds with significant antioxidant potential to tackle all biological routes associated with the parainflammatory syndrome secondary to oxidative stress.

## Author contributions

Conceptualization, R. D.-P., C. G.-V., and S. M.; methodology, C. M.-P.; formal analysis, C. M.-P. and S. M.; investigation R. D.-P., C. G.-V., and S. M.; resources, R. D.-P.; data curation, R. D.-P.; writing—original draft preparation, C. M.-P.; writing—review and editing, R. D.-P., C. G.-V., and S. M.; visualization, R. D.-P.; supervision, R. D.-P. and S. M.; project administration, R. D.-P.; funding acquisition, R. D.-P. and C. M.-P. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors declare that they have no conflicts of interest. Concepción Medrano-Padial is currently undergoing a traineeship at the European Food Safety Authority (EFSA) until 31 October 2025. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views/any official position or scientific works of the EFSA.

## Data availability

All data supporting the findings of this study are within the paper and are available from the corresponding author upon reasonable request.

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