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Transcriptional Modulation of the Nrf2 Antioxidant Pathway and Tight Junctions by a Plant Sterol Food Supplement in an Intestinal Inflammation Coculture Model

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ABSTRACT

There is growing evidence supporting the anti-inflammatory activity of plant sterols (PS) in preclinical models of intestinal inflammation. However, the underlying mechanisms driving these beneficial effects remain incompletely understood. This study investigates the transcriptional modulatory activity of a PS food supplement (PS-FS) on genes associated with the cellular antioxidant defense system and tight junction (TJ) proteins. A double-chamber coculture model with differentiated Caco-2 cells (apical) and RAW264.7 macrophages (basolateral) was used. Bioaccessible fraction (BF) of PS-FS was obtained following the INFOGEST 2.0 simulated gastrointestinal digestion. A 1/20 dilution of BF was applied to the apical chamber for 90 min, followed by lipopolysaccharide (LPS) stimulation (1 µg/mL, 24 h) on the basolateral side. The interaction between PS-FS and budesonide was also evaluated. The gene expression of Nrf2 (*NFE2L2*), glutathione S-transferase A1 (*GSTA1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and heme oxygenase 1 (*HMOX-1*) was quantified. Additionally, gene expression of TJ-related genes (*CLDN1*, *CLDN3*, *CLDN4*, *OCLN*, and *TJP1*) was quantified. Pretreatment with PS-FS significantly increased expression of *GSTA1* (26.8%) and *HMOX-1* (124.4%) compared to LPS + digestion blank. Furthermore, there was a trend toward increased expression of *NFE2L2* and *NQO1*. Similarly, PS-FS enhanced expression of *CLDN3* (33.0%), *CLDN4* (211.7%), *OCLN* (57.1%), and *TJP1* (98.9%). However, co-treatment of budesonide and PS-FS resulted in antagonistic interactions. These findings indicate that PS-FS modulates gene expression associated with antioxidant responses and intestinal barrier integrity-related pathways, suggesting to be further developed as a dietary strategy to support the management of inflammatory bowel disease

Practical Applications

This research supports the potential use of plant sterol-based food supplements in products aimed at supporting intestinal health. The results suggest that these supplements could help strengthen the gut barrier and support the body's natural antioxidant defenses during intestinal inflammation.

Abbreviations: ABTS, 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid); AAPH, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride; BF, bioaccessible fraction; CI, combination index; *CLDN1*, claudin-1 gene; *CLDN3*, claudin-3 gene; *CLDN4*, claudin-4 gene; *GSTA1*, glutathione S-transferase A1 gene; *HMOX-1*, heme oxygenase 1 gene; IBD, inflammatory bowel diseases; Keap1, kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; *NQO1*, NAD(P)H quinone dehydrogenase 1 gene; NF-κB, nuclear factor kappa B; *NFE2L2*, Nrf2 gene; Nrf2, nuclear factor erythroid 2–related factor 2; *OCLN*, occludin gene; ORAC, oxygen radical absorbance capacity; PS, plant sterols; PS-FS, plant sterol food supplement; TEAC, Trolox equivalent antioxidant capacity; TJ, tight junction; *TJP1*, zonula occludens-1 gene..

1 | Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic disorders of the gastrointestinal tract characterized by recurrent inflammatory episodes that significantly impact the quality of life of patients (Knowles et al. 2018; Guan 2019). Although pharmacological treatments such as glucocorticosteroids, immunosuppressants, and biologics are available, they may cause adverse effects and do not ensure sustained remission in all patients, highlighting the need for alternative therapeutic strategies with fewer side effects (Cai et al. 2021). In recent years, treatment approaches have expanded to include dietary interventions and bioactive compounds with anti-inflammatory properties as adjunctive strategies (Sasson et al. 2021). Among these, plant sterols (PS), natural phytochemicals found in plant-based foods, have attracted attention (Miszczuk et al. 2024). While they are well known for their cholesterol-lowering effects, growing evidence indicates that they also exert anti-inflammatory activity (Salehi et al. 2021). Preclinical studies, both in vitro (Moreno 2003; Cheon et al. 2006; Faubel et al. 2024) and in vivo (Islam et al. 2008; Mencarelli et al. 2009; Makran et al. 2025), have shown that PS inhibit key pro-inflammatory pathways such as nuclear factor kappa B (NF-κB), reduce pro-inflammatory cytokine production, and attenuate oxidative/nitrosative stress.

A recent study demonstrated that a PS food supplement (PS-FS), after simulated gastrointestinal digestion, exhibited stronger anti-inflammatory effects in a Caco-2/RAW264.7 coculture model than PS incorporated into food matrices (milk-based fruit beverage or rye bread), likely due to interactions with matrix components that may reduce PS bioactivity (Faubel et al. 2024). In a murine model of ulcerative colitis, PS-FS reduced rectal bleeding, restored the balance between pro-inflammatory cytokines (TNF-α, IL-6, IL-8) and the anti-inflammatory cytokine IL-10, and inhibited key enzymes (COX-2, iNOS) and transcription factors (NF-κB p65) (Makran et al. 2025). It also reduced tissue damage and increased occludin levels, a protein associated with intestinal barrier integrity. Despite the anti-inflammatory effects of PS-FS, a potential antagonistic interaction with budesonide, a commonly used corticosteroid, has been suggested and requires further investigation to clarify their combined effects (Faubel et al. 2024).

In addition to inflammation, oxidative stress and alterations in tight junction (TJ) proteins are also critical factors. Oxidative stress exacerbates inflammation by damaging cellular components, while disruption of TJ proteins increases intestinal permeability and further promotes inflammation (Zhu and Li 2012; Lee 2015). Therefore, current research focuses on therapeutic approaches that restore antioxidant signaling pathways and enhance TJ protein gene expression. In this context, the transcription factor Nrf2 has emerged as a promising therapeutic target, as it regulates antioxidant enzyme expression, reduces oxidative stress, and promotes TJ protein gene expression, thereby potentially contributing to intestinal barrier restoration (Wen et al. 2019; B. Li et al. 2023).

Based on this background, the aim of the present study was to evaluate whether PS-FS can modulate gene expression associated with the Nrf2-antioxidant enzyme-TJ axis in an intestinal inflammation model based on the coculture of Caco-2 intestinal cells and RAW264.7 macrophages. This in vitro model is widely used to study intestinal inflammatory responses (Tanoue et al. 2008) and has been previously validated through comparison with a murine model of DSS-induced colitis (Makran et al. 2025). To the best of our knowledge, this is the first study to investigate these molecular responses using the bioaccessible fraction (BF) of PS-FS in this experimental model. Although the interaction between PS-FS and budesonide has been previously evaluated at the functional level (Faubel et al. 2024), the present work provides novel insight at the transcriptional level.

2 | Materials and Methods

2.1 | Reagents

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Folin–Ciocalteu reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), lipopolysaccharide (LPS) from *Escherichia coli* O127:B8, potassium persulfate, sodium carbonate, and sodium fluorescein were sourced from Merck LifeScience S.L.U. (Madrid, Spain). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). ReliaPrep RNA miniprep system kit was provided by Promega (Madison, WI, USA), while the TaqMan reverse transcription kit and PowerUp SYBR Green Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). D-MEM + GlutaMAX (4.5 g/L glucose), MEM nonessential amino acids (MEM NEAA) solution (100x), HEPES buffer solution (1 M), antibiotic solution (10,000 U/mL penicillin and 10,000 µg/mL streptomycin), antimycotic solution (250 µg/mL amphotericin B), and fetal bovine serum (FBS) were all obtained from Gibco (Scotland, UK). Ultrapure water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 | Sample

PS-FS is composed of a commercially available microencapsulated unesterified PS ingredient (Lipophytol P Dispersable; Lipofoods, Spain), with a PS purity of 86.8% (w/w). The PS profile was characterized by gas chromatography coupled with a flame ionization detector in a previous study (Faubel et al. 2024). The identified PS profile includes (%, w/w) β-sitosterol (60.7 ± 0.8), sitostanol (15.9 ± 0.7), campesterol (6.7 ± 0.3), campestanol (2.0 ± 0.1), Δ^7-stigmastenol (0.51 ± 0.02), stigmasterol (0.48 ± 0.02), Δ^7-avenasterol (0.33 ± 0.01), $\Delta^{5,24}$-stigmastadienol (0.247 ± 0.003), and Δ^5-avenasterol (0.085 ± 0.004).

2.3 | In Vitro Gastrointestinal Digestion

To simulate gastrointestinal conditions during the exposure of the cell culture to the PS, the PS-FS was subjected to simulated gastrointestinal digestion using the adapted INFOGEST 2.0 harmonized method specifically tailored for PS (Makran et al. 2022). The reagents and their preparation are described by Brodkorb

et al. (2019) and Makran et al. (2022). Initially, the PS-FS was prepared by reconstituting 2.3 g of PS-FS (containing 2 g of PS based on its purity) in 200 mL of water, mimicking the typical method for ingesting powdered supplements. Then, 5 mL of this suspension (or 5 mL of ultrapure water as a digestion blank) was mixed with 3.5 mL of simulated salivary fluid and shaken for 1 min. Next, 0.5 mL of α-amylase solution (to achieve a final concentration of 75 U/mL), 25 μL of 0.3 M calcium chloride, and 975 μL of ultrapure water were added to reach a final volume of 10 mL. The mixture was then incubated in a shaking bath at 37°C and 95 rpm for 2 min to complete the oral phase. For the gastric phase, 7.5 mL of simulated gastric fluid and 5 μL of 0.3 M calcium chloride were added to the oral digesta and manually mixed for 1 min. Then, 0.98 mL of rabbit gastric extract solution and 0.62 mL of pepsin solution were added (to achieve final concentrations of 60 U/mL of lipase and 2000 U/mL of pepsin). The pH was adjusted to 3, and ultrapure water was added to reach a final volume of 20 mL. The mixture was then incubated in a shaking bath at 37°C with agitation for 2 h. To simulate the intestinal phase, 11 mL of simulated intestinal fluid, 5 mL of pancreatin solution (to achieve a final concentration of 100 U/mL), 40 μL of 0.3 M calcium chloride, 2.5 mL of bovine bile extract solution (to achieve a final concentration of 10 mM bile salts), and 0.1 mL of cholesterol esterase solution (to achieve a final concentration of 0.075 U/mL) were added to the gastric digesta. The mixture was manually mixed for 1 min, adjusted to pH 7, and ultrapure water was added to bring the volume to 40 mL. It was then incubated in a shaking bath at 37°C with agitation for 2 h. The resulting digesta was centrifuged (3100 × *g*, 90 min, 4°C), and the supernatant, representing the BF, was collected.

2.4 | Cell Lines

Human adenocarcinoma colonic epithelial (Caco-2, passage 32–40) cells and murine macrophage (RAW264.7, passage 30–36) were obtained from the American Type Culture Collection (HTB-37 and TIB-71, respectively; Rockville, MD, USA). For cell growth, they were cultured in 75 cm^2 flasks (Corning Falcon) using D-MEM + GlutaMAX supplemented with 10% (v/v) FBS, 1% (v/v) MEM NEAA, 1% (v/v) antibiotic solution, 0.2% (v/v) antimycotic solution, and 1% (v/v) HEPES. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% relative humidity.

2.5 | Cell Coculture Model

The coculture of Caco-2 cells and RAW264.7 macrophages was employed as a model of intestinal inflammation following the protocol previously described (Faubel et al. 2024). Caco-2 cells were seeded at a density of 3.75×10^5 cells per insert in the Transwell (membrane area of 4.67 cm^2, pore size of 0.4 μm; Corning CoStar Corp., Cambridge, MA, USA) and cultured to a differentiated state over 15 days, confirmed by a transepithelial electrical resistance of 800 Ω × cm^2. One day prior to reaching the differentiation of Caco-2 cells, RAW264.7 macrophages were seeded at a density of 8.5×10^5 cells per well in a six-well plate and maintained for 24 h. Following this, Transwell inserts containing the differentiated Caco-2 cells were placed into the six-well plate with RAW264.7 macrophages. Then, a 1/20-dilution (v/v) of the BF of PS-FS or digestion blank was added to the apical compartment and incubated for 90 min. This dilution was selected based on previous optimization experiments (Faubel et al. 2024) and was confirmed in the present study to be noncytotoxic by MTT assay, following the methodology described by Cilla et al. (2022). This ensured that the observed effects were not influenced by cell damage. At this dilution, the final concentration of PS in the BF was 13 μM (Faubel et al. 2024). To assess the interaction between budesonide (a drug widely used in the treatment of IBD) and PS-FS, budesonide (1 μM) was introduced into the apical compartment, both as an independent treatment and in combination with the BF of PS-FS. This concentration was selected based on previous studies using this coculture model (Faubel et al. 2024), as well as on the original model described by Tanoue et al. (2008), where it is recommended as a pharmacological reference condition due to its anti-inflammatory activity without compromising cell viability. After incubation, the apical medium was replaced with fresh culture medium, and a pro-inflammatory response was induced by adding 1 μg/mL of LPS to the basolateral chamber for 24 h. The assay was performed in triplicate for each experimental condition.

2.6 | Gene Expression Analysis

The expression of genes involved in antioxidant defense and TJ in differentiated Caco-2 cells was assessed using qPCR. Gene-specific primers were designed with Primer-BLAST software (NCBI), following standard criteria for PCR product size (150–200 base pairs) and melting temperature (57°C–60°C). Primer specificity was verified through post-amplification melting-curve analysis, and primer efficiency was confirmed by constructing a standard curve from serial cDNA dilutions. Sequences of the primers used for the target and reference genes, along with their respective amplification efficiencies, are detailed in the Table 1.

The gene expression analysis was conducted as described in a previous study (Makran et al. 2023). Briefly, the culture medium was removed, 1 mL of TRIzol reagent was added to the insert and cells were scraped off (Sarstedt, Nümbrecht, Germany). The resulting cell lysate was mixed with 200 μL of chloroform and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was then combined with 170 μL of isopropanol, and the RNA was purified using the ReliaPrep RNA Miniprep System kit. RNA concentration and purity were assessed using a Thermo Scientific NanoDrop Lite, with acceptable purity ratios of A_{260}/A_{280} (1.8–2.0) and A_{260}/A_{230} (≥ 2.0). The purified RNA was reverse transcribed into cDNA using the TaqMan Reverse Transcription kit, following the manufacturer's protocol, with a final RNA concentration of 30 ng/μL. The reaction conditions were set as follows: 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, followed by stabilization at 16°C. Subsequent amplification reactions were carried out in MicroAmp Fast Optical 96-Well Reaction Plates using SYBR Green detection chemistry on a StepOne Plus Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). Each reaction, with a final volume of 10 μL, contained 45 ng of template cDNA, 2 μL of primer pair solution (900 nM final concentration for each primer), and 5 μL of PowerUp SYBR Green Master Mix. PCR cycling protocol included an initial hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 15 s.

TABLE 1 | Sequence of primers of the evaluated genes and their efficiency.

Gene	Primer sequence (5′→ 3′)		Efficiency (%)
	Forward	Reverse	
NFE2L2	CGGTATGCAACAGGACATTG	AGAGGATGCTGCTGAAGGAA	107
GSTA1	GTGCAGACCAGAGCCATTC	TCACCCAAATCTGCTATACCTTC	98
HMOX-1	CCAGGCAGAGAATGCTGAGT	GTAGACAGGGGCGAAGACTG	104
NQO1	CGCAGACCTTGTGATATTCCAGT	TCCTATGAACACTCGCTCAAACC	107
CLDN1	CACCGTCTGTGTTTGAGCA	CAAACCACCGCTTACAGATG	107
CLDN3	AACCTGCATGGACTGTGAAA	GGTCAAGTATTGGCGGTCAC	112
CLDN4	AACCTGTCCCCGAGAGAGA	GCAAGTGTGAGCAGACCAGT	110
OCLN	AAGGGAGCCTGTCTATTGGAA	AAAAAGCATGCAACTTGAAAGA	86
TJP1	AAATTTAACTAATGTCAGACTGGAGGA	TCAGCTTGTGGTGAGTAAGAGG	95
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	104

All reactions were conducted in triplicate. Gene expression fold changes were determined using the $2^{-\Delta\Delta Cq}$ method, using the *ACTB* gene as reference after verifying its consistent expression across all experimental conditions.

2.7 | Combination Index

The potential interaction between PS-FS and budesonide in modulating target gene expression was evaluated by calculating the combination index (CI). The CI was calculated based on the comparison between the effects of combined and individual treatments at a single concentration, following the general principles described by Chou (2010), as applied in the study by Álvarez-Sala et al. (2018), with the following formula: CI = (% PS-FS + budesonide)/(% PS-FS + % budesonide), where "% PS-FS" and "% budesonide" denote the percentage increase in gene expression from individual treatments with PS-FS and budesonide, respectively. "% PS-FS + budesonide" represents the percentage increase achieved with the combined treatment. A CI value less than, equal to, or greater than 1 indicates antagonism, additivity, or synergy, respectively. This calculation was performed exclusively in cases where both the individual treatments and the co-treatment resulted in statistically significant effects.

2.8 | Antioxidant Capacity

The antioxidant capacity of the PS-FS, suspended in ultrapure water at 50 g/L (concentration that ensures adequate aqueous dispersion of the supplement), and its BF was assessed using three distinct methods, each evaluating a different mechanism of antioxidant action: oxygen radical absorbance capacity (ORAC) for hydrogen atom transfer, Folin–Ciocalteu reducing capacity for electron transfer, and Trolox equivalent antioxidant capacity (TEAC) for a mixed mode (Prior et al. 2005; Munteanu and Apetrei 2021). The activity of the digestion blank was evaluated to subtract any antioxidant activity from the reagents used in the simulated gastrointestinal digestion. Each sample was analyzed in triplicate.

2.8.1 | ORAC Method

The ORAC assay was conducted following established protocols (Cilla et al. 2011). Briefly, 80 µL of sodium fluorescein (0.015 mg/mL), 80 µL of the samples (PS-FS suspension or BF diluted at 1/100 (v/v) in 75 mM phosphate buffer, pH 7.4), and 40 µL of AAPH (120 mg/mL) were added to a white multi-well plate. Fluorescence intensity was recorded every 5 min for 90 min (λ_{exc} = 485 nm, λ_{em} = 520 nm) using a multi-well spectrophotometer (Victor^3 Multi-Label Counter, Perkin-Elmer, Waltham, MA, USA). The area under the curve was calculated, and results were expressed as µmol Trolox/100 g PS-FS, using a Trolox standard (20 µM) for comparison.

2.8.2 | Folin–Ciocalteu Reducing Capacity

The Folin–Ciocalteu reducing capacity was measured using a procedure previously described with some modifications (Cilla et al. 2011). A 100 µL sample was mixed with 3 mL of a 2% (w/v) sodium carbonate solution and 100 µL of 1 N Folin–Ciocalteu reagent. The mixture was incubated at room temperature in the dark for 1 h. Absorbance was then measured at 765 nm using a UV-VIS spectrophotometer (Lambda 365, Perkin-Elmer, Waltham, Massachusetts, USA). Results were expressed as gallic acid equivalents (GAE)/100 g of PS-FS, determined using a calibration curve with a gallic acid standard (10–300 mg/L).

2.8.3 | TEAC Method

The TEAC assay was performed following previously published protocols (Cilla et al. 2011). ABTS stock solution was prepared by mixing 6.9 mM ABTS with 2.4 mM potassium persulfate and allowing it to react in the dark at room temperature for 12–16 h. The stock solution was then diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm (30°C). Absorbance of 2 mL of the diluted ABTS solution was measured by an UV-VIS spectrophotometer (Lambda 365, Perkin-Elmer, Waltham, Massachusetts, USA) before and after incubation for 3 min with 100 µL of the samples (PS-FS suspension or BF diluted

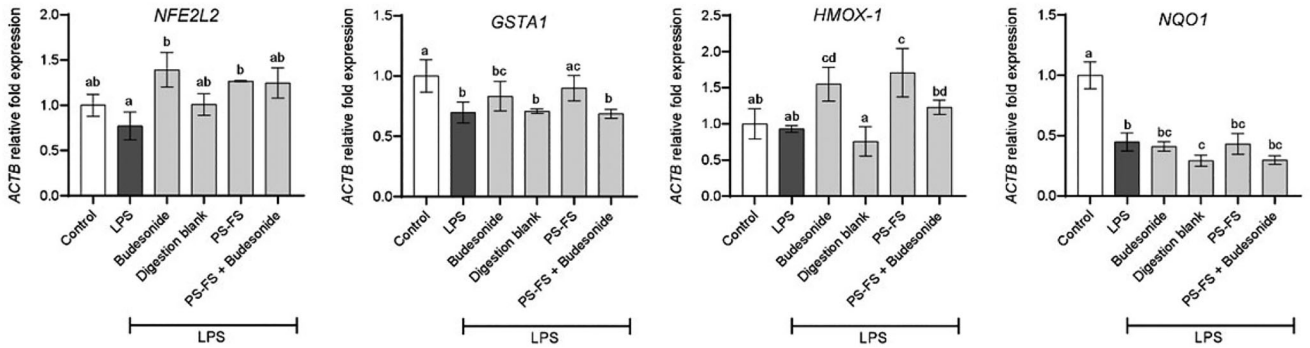

FIGURE 1 | Relative expression of genes involved in the cellular antioxidant defense system was evaluated in Caco-2 cells using a two-compartment model comprising differentiated Caco-2 cells (apical) and RAW264.7 macrophages (basolateral). The bioaccessible fraction of a plant sterol food supplement (PS-FS) diluted 1/20 (v/v), budesonide (1 μM), or their combination was added to the apical compartment for 90 min, followed by lipopolysaccharide (LPS) treatment at 1 μg/mL for 24 h in the basolateral compartment. Data are expressed as mean ± standard deviation ($n = 9$). Statistically significant differences ($p < 0.05$) between conditions for the same gene are indicated by different letters. The gene encoding Nrf2 (*NFE2L2*) was evaluated, as well as the genes encoding the enzymes glutathione S-transferase A1 (*GSTA1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and heme oxygenase 1 (*HMOX-1*).

1/2, v/v) or Trolox standards (100–1000 μM). The percentage of inhibition was calculated, and the results were expressed as μmol of Trolox/100 g of PS-FS.

2.9 | Statistical Analysis

Data are expressed as mean ± standard deviation. All experiments were performed using three independent biological replicates, each analyzed in triplicate (technical replicates), resulting in a total of $n = 9$ measurements. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test or a *T*-test to determine significant differences ($p < 0.05$). Statgraphics Plus 5.1 software (Statpoint Technologies Inc., Warrenton, VA, USA) was utilized for all analyses.

3 | Results

3.1 | Improved Expression of Cellular Antioxidant System Genes

To assess the impact of PS-FS on the Nrf2 signaling pathway and its target enzymes, an analysis of the expression levels of the Nrf2 gene (*NFE2L2*), along with the genes encoding its target enzymes: glutathione S-transferase A1 (*GSTA1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and heme oxygenase 1 (*HMOX-1*), was performed. These enzymes play crucial roles in cellular defense mechanisms against oxidative stress, and their upregulation is suggestive of transcriptional modulation associated with the Nrf2 pathway, providing insight into the potential of PS to modulate antioxidant responses through the Nrf2 signaling cascade. LPS treatment in the cell coculture model resulted in a statistically significant ($p < 0.05$) downregulation of *GSTA1* (30.3%) and *NQO1* (55.2%) gene expression in Caco-2 cells (Figure 1).

Although reductions in *NFE2L2* (1.00 ± 0.12 vs. 0.77 ± 0.15 fold-change) and *HMOX-1* (1.00 ± 0.21 vs. 0.93 ± 0.05 fold-change) gene expression were observed compared to control cells, these changes did not achieve statistical significance ($p > 0.05$). In general, pretreatment with the digestion blank did not modify the impact of LPS on gene expression. However, it did exacerbate the reduction of *NQO1* expression compared to cells exposed only to LPS (70.82 vs. 55.2%, respectively), suggesting a potential interaction between the blank and the LPS-induced response. On the other hand, pretreatment with budesonide, used as a positive control, was not able to restore the expression of *NQO1* that had been significantly reduced by LPS. Nevertheless, a tendency to increase the expression of the *GSTA1* gene was observed against cells exposed to LPS (0.83 ± 0.12 vs. 0.70 ± 0.09 fold-change), although without reaching statistical significance ($p > 0.05$). Importantly, budesonide significantly upregulated the expression of *NFE2L2* and *HMOX-1* (80.4% and 66.3%, respectively), indicating a partial protective effect against oxidative stress. Moreover, PS-FS pretreatment significantly increased the expression of *GSTA1* and *HMOX-1* genes (26.8% and 124.4%, respectively) compared to the LPS + digestion blank ($p < 0.05$). *NFE2L2* expression showed a nonsignificant trend toward increase from 1.01 ± 0.12 (LPS + digestion blank) to 1.27 ± 0.01 fold-change (PS-FS-treated cells; $p > 0.05$). A similar nonsignificant trend ($p > 0.05$) was observed for *NQO1*, with higher expression in PS-FS-treated cells (0.43 ± 0.09 fold-change) compared to the LPS + digestion blank (0.29 ± 0.05 fold-change).

Furthermore, trends toward increased expression of *NFE2L2* (1.01 ± 0.12 vs. 1.27 ± 0.01 fold-change) and *NQO1* (0.29 ± 0.05 vs. 0.43 ± 0.09 fold-change) were observed in PS-FS-treated cells (vs. LPS + digestion blank), although without reaching statistical significance ($p > 0.05$). These findings support the hypothesis that PS-FS may modulate components of the Nrf2 signaling pathway and associated antioxidant defenses.

3.2 | Restoration of the Expression of Genes Encoding TJ

The TJ proteins, essential for maintaining the integrity of the intestinal barrier, are frequently compromised during inflammatory states. Therefore, understanding how different treatments affect the expression of these genes provides valuable insights into

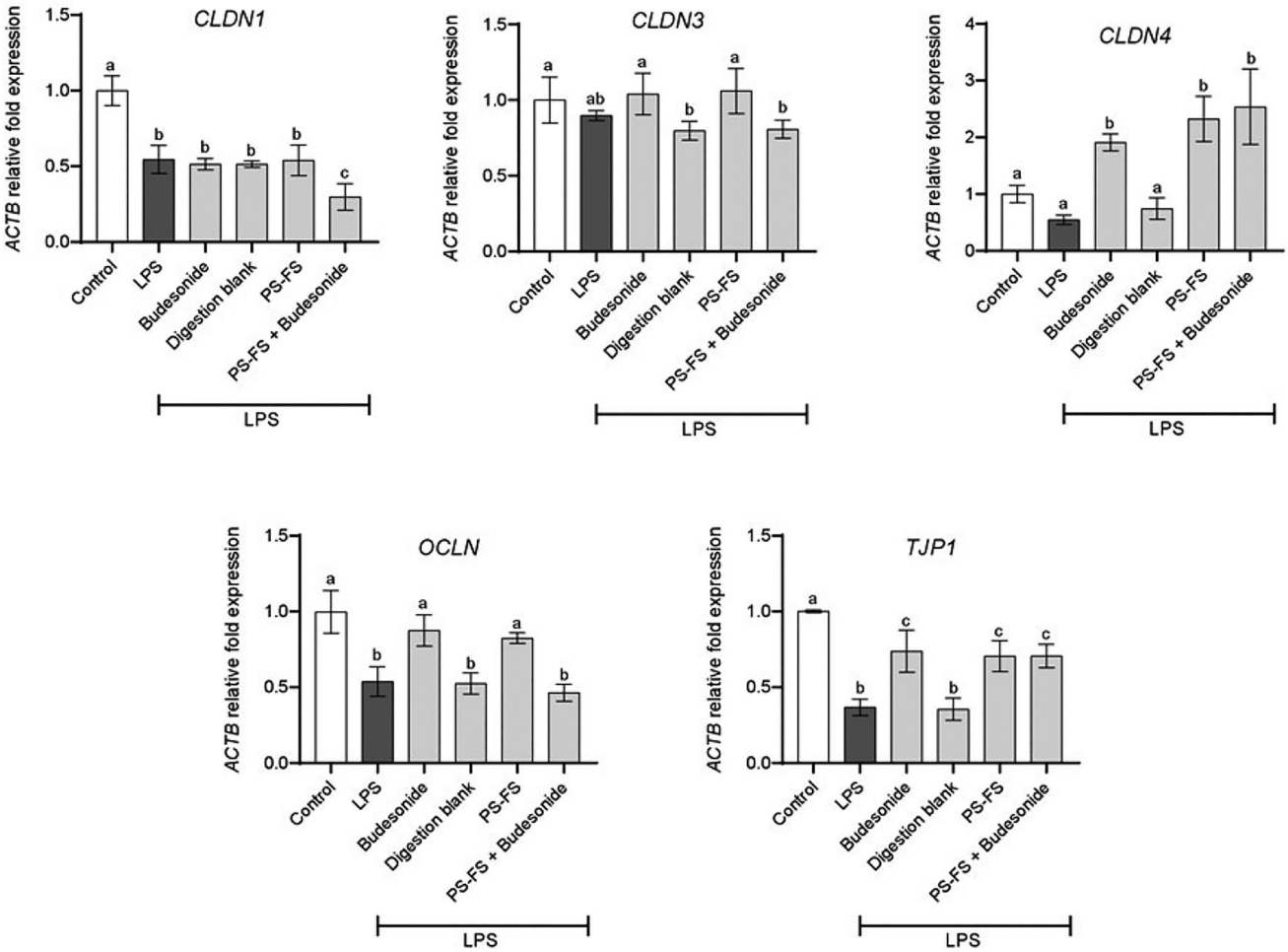

FIGURE 2 | Relative expression of tight junction genes was evaluated in Caco-2 cells using a two-compartment model comprising differentiated Caco-2 cells (apical) and RAW264.7 macrophages (basolateral). The bioaccessible fraction of a plant sterol food supplement (PS-FS) diluted 1/20 (v/v), budesonide (1 μM), or their combination was added to the apical compartment for 90 min, followed by lipopolysaccharide (LPS) treatment at 1 μg/mL for 24 h in the basolateral compartment. Data are expressed as mean ± standard deviation ($n = 9$). Statistically significant differences ($p < 0.05$) between conditions for the same gene are indicated by different letters. The genes encoding the tight junction proteins claudins-1, -3 and -4 (*CLDN1*, *CLDN3*, and *CLDN4*, respectively), occludin (*OCLN*), and zonula occludens-1 (*TJP1*) were evaluated.

their potential to preserve or restore intestinal barrier function under inflammatory environment. In the present study, the gene expression of TJ—claudins-1, -3, and -4 (*CLDN1*, *CLDN3*, and *CLDN4*, respectively), occludin (*OCLN*), and zonula occludens-1 (*TJP1*)—was evaluated in Caco-2 cells (Figure 2).

In this sense, exposure to LPS significantly reduced the expression of *CLDN1* (45.4%), *OCLN* (46.2%), and *TJP1* (63.3%), in comparison to control cells ($p < 0.05$). While gene expression of *CLDN3* (1.00 ± 0.15 vs. 0.90 ± 0.04 fold-change) and *CLDN4* (1.00 ± 0.15 vs. 0.55 ± 0.09 fold-change) also showed a trend toward reduced expression, these changes were not statistically significant ($p > 0.05$). Digestion blank had no effect on the expression of any of the TJ genes evaluated, indicating that it did not interfere with the LPS-induced alterations. Budesonide pretreatment effectively enhanced the expression of *CLDN4* (247.3%), *OCLN* (63.0%), and *TJP1* (100.8%), compared to LPS-treated cells ($p < 0.05$). Similarly, the PS-FS pretreatment improved the expression of *CLDN3* (33.0%), *CLDN4* (211.7%), *OCLN* (57.1%), and *TJP1* (98.9%) genes, compared to cells exposed to LPS + digestion blank ($p < 0.05$), highlighting its potential in enhancing TJ gene expression and possibly mitigating LPS-induced barrier dysfunction.

3.3 | Antagonistic Interaction Between Budesonide and PS-FS

According to the results described above, a variable pattern was observed in the effects of budesonide and PS-FS on the expression of antioxidant defense genes. While budesonide enhanced the gene expression of *NFE2L2* and *HMOX-1*, PS-FS positively modulated the expression of *GSTA1* and *HMOX-1*. Notably, in the case of *HMOX-1*, which both agents are capable of modulating, PS-FS seems to demonstrate greater effectiveness compared to the drug budesonide (1.71 ± 0.33 vs. 1.55 ± 0.24 fold-change, respectively), although the difference was not statistically significant ($p > 0.05$). On the other hand, it is important to note that while exposure to budesonide alone significantly increased *NFE2L2* gene expression, its co-incubation with PS-FS negated this effect, as

TABLE 2 | Antioxidant capacity of the plant sterol food supplement (PS-FS) in its undigested form and bioaccessible fraction after in vitro gastrointestinal digestion.

Undigested PS-FS	Bioaccessible PS-FS	Bioaccessibility (%)
Folin–Ciocalteu reducing capacity (mg GAE/100 g PS-FS)		
388.93 ± 33.45	3.94 ± 0.34*	1.02 ± 0.09
TEAC (μmol Trolox/100 g PS-FS)		
271.00 ± 26.04	43.55 ± 3.80*	16.07 ± 1.40
ORAC (μmol Trolox/100 g PS-FS)		
1020.04 ± 89.41	383.64 ± 29.76*	33.31 ± 1.59

Note: Values are presented as mean ± standard deviation ($n = 3$).

Abbreviations: ORAC, oxygen radical absorbance capacity; TEAC, Trolox equivalent antioxidant capacity.

* indicates a statistically significant reduction compared to the undigested sample ($p < 0.05$).

the co-treatment did not show statistically significant differences compared to the LPS + digestion blank ($p > 0.05$). Similarly, modulatory effect of PS-FS on *GSTA1* expression was abolished when combined with budesonide, resulting in expression levels comparable to the LPS + digestion blank. In the case of *HMOX-1* gene, co-incubation showed an upregulation compared to the LPS + digestion blank. However, the calculated CI indicated an antagonistic effect (CI, 0.35 ± 0.04), indicating that the combined treatment was less effective than the sum of the individual effects of each agent.

Regarding the modulatory effects of budesonide and PS-FS on the expression of genes encoding TJ proteins, both agents were effective in enhancing the expression of *CLDN4*, *OCLN*, and *TJP1*. However, PS-FS showed greater efficacy than budesonide by also increasing *CLDN3* gene expression, compared with budesonide, which did not significantly affect *CLDN3* expression. In the co-treatment scenario, the positive effect on *OCLN* expression was nullified, despite significant effects from the individual treatments. In addition, although the co-treatment sustained the beneficial effects on *CLDN4* and *TJP1* expression, the CI indicated an antagonistic interaction (CI, 0.66 ± 0.11 and 0.40 ± 0.02, respectively), as the observed increase was comparable to that induced by the individual treatments. Moreover, the positive effect of PS-FS on *CLDN3* gene expression was statistically diminished in the presence of budesonide (1.06 ± 0.15 vs. 0.81 ± 0.06 fold-change, respectively; $p < 0.05$).

3.4 | Antioxidant Capacity of PS-FS and Effect of Simulated Gastrointestinal Digestion

The antioxidant activity of the PS-FS was assessed in both its undigested form and after being subjected to an in vitro digestion process (Table 2). The results demonstrated a significant reduction in antioxidant capacity following simulated gastrointestinal digestion. In the case of Folin–Ciocalteu reducing capacity, undigested sample exhibited an antioxidant capacity of 388.9 mg GAE/100 g PS-FS; however, this activity dramatically decreased in the BF to 3.9 mg GAE/100 g PS-FS ($p < 0.05$), representing a bioaccessibility of 1%. Similar results were observed with the TEAC assay, where the undigested form showed a value of 271 μmol Trolox per 100 g of PS-FS, which was significantly reduced to 43.5 μmol Trolox/100 g ($p < 0.05$) after simulated digestion (bioaccessibility of 16.1%). Accordingly, the ORAC assay results also reflected a substantial reduction in antioxidant capacity post-digestion, with the undigested sample showing a capacity of 1020 μmol Trolox/100 g. This value decreased to 383 μmol Trolox/100 g ($p < 0.05$) in the BF, yielding a bioaccessibility of 33.3%.

4 | Discussion

PS are a diverse group of bioactive compounds known for their blood cholesterol-lowering effects (Salehi et al. 2021). Recent research has uncovered additional health benefits of PS, particularly their antioxidant and anti-inflammatory properties (Miszczuk et al. 2024). Numerous preclinical studies have demonstrated that PS can modulate the inflammatory response in the intestinal tract, leading to improvements in biochemical parameters, histopathological outcomes, and clinical symptoms (Moreno 2003; Cheon et al. 2006; Islam et al. 2008; Mencarelli et al. 2009; Faubel et al. 2024; Makran et al. 2025). These studies have primarily focused on the cellular regulatory pathways involved in the inflammatory response to elucidate the mechanisms by which PS alleviate intestinal inflammation. However, IBD are very complex, with multiple deregulated cellular signaling pathways, with oxidative stress being a crucial factor in the development of these conditions. Therefore, current research aims at identifying bioactive compounds capable of modulating the signaling pathways involved in oxidative stress responses, offering new therapeutic approaches for IBD (Jarmakiewicz-Czaja et al. 2023). Notably, activating the transcription factor Nrf2 can enhance the expression of antioxidant enzymes, significantly reducing cellular oxidative stress and potentially improving intestinal inflammation. Additionally, Nrf2 activation has been shown to enhance the expression of genes encoding TJ proteins, which is particularly important since their levels are significantly reduced in the context of intestinal inflammation (Wen et al. 2019). Due to the lack of information on this topic, this study aimed at investigating the mechanism of action of PS in the context of intestinal inflammation, focusing on the gene expression of the transcription factor Nrf2, its target enzymes, and TJ proteins. An in vitro model of intestinal inflammation was used, involving the coculture of intestinal Caco-2 cells and RAW264.7 macrophages. The results showed that PS-FS is able to positively modulate the transcription of antioxidant genes, showing a significant increase

in the gene expression of *GSTA1* and *HMOX-1*, with nonsignificant trends toward higher expression of *NFE2L2* and *NQO1*. These findings are consistent with the potential involvement of the Nrf2-dependent signaling pathway, which may be associated with improved cellular antioxidant responses.

This upregulation of antioxidant genes, in line with previous findings demonstrating inhibition of the pro-inflammatory NF-κB-COX-2-PGE_2 pathway, along with a concomitant reduction in the secretion of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) and markers of nitrosative/oxidative stress (iNOS/NO and ROS) (Faubel et al. 2024; Makran et al. 2025). Altogether, these data support the hypothesis that PS-FS exerts its anti-inflammatory effects, at least in part, through modulation of the Nrf2 signaling cascade at transcriptional level. The modulation of the Nrf2 signaling pathway by numerous bioactive compounds is well-documented (M. Li et al. 2019). Under normal conditions, Nrf2 is sequestered in the cytoplasm by its inhibitor, Kelch-like ECH-associated protein 1 (Keap1), which binds to Nrf2, promoting its ubiquitination and subsequent proteasomal degradation (Bellezza et al. 2018). Certain bioactive compounds have been shown to bind to Keap1, leading to the release of Nrf2 (M. Li et al. 2019). This allows Nrf2 to translocate to the nucleus and bind to antioxidant response elements (AREs) in the promoter regions of target genes, leading to their transcriptional activation. In this context, a previous study demonstrated through computational molecular docking analysis that several PS (β-sitosterol, stigmasterol, campesterol, and 28-isofucosterol) can directly inhibit Keap1 (Syahputra et al. 2022). Therefore, considering previous evidence together with the results of the present study, PS may be associated with the modulation of antioxidant gene expression, potentially involving Nrf2-related pathways. In this context, previous molecular docking studies have suggested that certain PS may interact with Keap1; however, this mechanism was not experimentally evaluated in the present work.

On the other hand, the results of the present study showed that PS-FS enhanced the expression of genes encoding several TJ proteins, including *CLDN3*, *CLDN4*, *OCLN*, and *TJP1*. These findings are consistent with previous studies showing increased in occludin protein levels following pretreatment with PS-FS in a coculture of Caco-2 cells and RAW264.7 macrophages exposed to LPS, as well as in a murine colitis model (Makran et al. 2025). This suggests that PS-FS may help restore TJ integrity and protect against LPS-induced barrier dysfunction in this in vitro model. However, confirmation of Nrf2 pathway activation would require additional evidence, such as assessment of Nrf2 nuclear translocation, protein expression levels, or ARE-driven transcriptional activity. However, functional measurements of barrier integrity were not included in the present study, and further work is needed to confirm these effects at the protein and functional levels.

On the other hand, it is well established that oxidative stress negatively affects the expression and function of TJ proteins, contributing to epithelial barrier dysfunction (Rao 2008). In this context, the transcriptional upregulation of antioxidant defense genes observed in the present study, potentially involving Nrf2-related pathways, may be associated with the regulation of TJ-related gene expression under inflammatory conditions. In addition, it is important to distinguish between the chemical and biological aspects of the antioxidant response. The antioxidant capacity measured by TEAC and ORAC assays provides complementary information on the redox properties of the PS-FS. However, these chemical assays do not necessarily reflect cellular antioxidant mechanisms or signaling pathway modulation. In contrast, previous studies have demonstrated a reduction in oxidative stress at the functional level, including decreased intracellular ROS levels and MPO activity (Faubel et al. 2024; Makran et al. 2025). In this framework, the transcriptional upregulation of antioxidant-related genes observed in the present study may contribute to these previously reported biological effects.

The beneficial effect observed with the pretreatment of the BF of PS-FS would result from the action of bioaccessible PS remaining after the in vitro gastrointestinal digestion, which has been reported in a previous study at 8.1% (Faubel et al. 2024). This bioaccessibility value was similar to that obtained in a food matrix, such as PS-enriched fruit milk-based beverage (Makran et al. 2022).

The present study also aimed at evaluating the modulatory effects of PS-FS in combination with the drug budesonide, revealing an antagonistic interaction between the two agents. Previous research has shown that the combination of PS-FS and budesonide produces mainly an antagonistic effect (except in TNF-α and IL-8 where an additive effect was observed) in terms of improvement of biochemical parameters such as secretion of inflammatory mediators (IL-6, NO, and PGE_2), levels of pro-inflammatory enzymes (COX-2 and iNOS), intracellular ROS production, and nuclear translocation of NF-κB p65 (Faubel et al. 2024). In this study, the antagonistic interaction showed that the effects of the co-treatment were equivalent to those observed with the agents alone. In the same way, the current research obtained similar findings regarding the gene expression of *HMOX-1*, *CLDN3*, *CLDN4*, and *TJP1*. However, the most surprising findings of the study were that while individual treatments were effective, co-treatment had no beneficial effect on the gene expression of *NFE2L2*, *GSTA1*, and *OCLN*. As a whole, these data indicate that co-treatment is not advantageous, as it either fails to produce an additive or synergistic effect or completely cancels out the beneficial effects observed with individual treatments. These antagonistic effects may be explained by interactions between the signaling pathways activated by both agents. Glucocorticoids such as budesonide exert their effects through activation of the glucocorticoid receptor, which regulates gene transcription of inflammatory mediators. However, evidence suggests that crosstalk may occur between glucocorticoid receptor signaling and Nrf2-related pathways. In particular, glucocorticoid receptor activation has been reported to repress Nrf2-mediated antioxidant responses by inhibiting its transcriptional activity, including through epigenetic mechanisms affecting histone acetylation (Alam et al. 2017). In this context, the simultaneous modulation of redox-sensitive pathways by PS-FS and activation of glucocorticoid receptor signaling by budesonide may lead to interference between these regulatory systems.

From a translational perspective, the interaction observed between PS-FS and budesonide highlights the complexity of combining nutraceuticals with pharmacological treatments. While there is increasing evidence supporting the role of nutraceuti-

cals in modulating inflammation and oxidative stress in IBD, studies specifically addressing their interaction with conventional therapies remain limited (Ashrafpour et al. 2025). Therefore, the present findings contribute to a still underexplored area and highlight the need for further studies to better understand nutraceutical–drug interactions in IBD. This finding also underscores the complexity of combining pharmacological agents with dietary bioactive compounds in multifactorial diseases such as IBD. It also highlights the need to carefully considering potential interactions when designing combination strategies.

Despite the relevance of these findings, some limitations of the present study should be acknowledged. First, the conclusions are primarily based on gene expression analysis, without direct assessment at the protein or functional level within the same experimental framework. However, it is important to note that the biological activity of PS-FS has been previously validated at the protein and functional levels, both in vitro and in vivo, including the modulation of inflammatory pathways and the improvement of intestinal barrier integrity (Faubel et al. 2024; Makran et al. 2025). Therefore, the transcriptional modulation observed in the present study should be interpreted as providing further mechanistic insight into these previously established effects. Nevertheless, additional proteomic or functional analyses would be valuable to confirm whether the observed changes at the gene expression level are translated into protein-level modulation. This includes the assessment of Nrf2 nuclear translocation, antioxidant enzyme levels, as well as barrier functionality (e.g., TEER or permeability assays). Second, although the in vitro coculture model represents a well-established approach to study intestinal inflammation, it does not fully reproduce the complexity of the in vivo environment. In this regard, previous studies have already demonstrated the efficacy of PS-FS in murine models of intestinal inflammation (Makran et al. 2025). However, further research using additional experimental models of intestinal inflammation would be useful to broaden the understanding of its biological effects and enhance the translational relevance of these findings.

In addition, it should be noted that the PS composition was characterized both before and after simulated gastrointestinal digestion. However, the specific molecular species generated during digestion were not identified, and approaches such as metabolite profiling or network pharmacology analysis were not included. These strategies could provide a more comprehensive understanding of the bioactive compounds responsible for the observed effects and their potential molecular targets. Finally, the INFOGEST in vitro digestion model, while widely used, does not fully replicate in vivo conditions, particularly regarding the influence of the gut microbiota and the mucus layer, which may affect the bioavailability and biological activity of PS. Addressing these aspects in future studies would provide a more comprehensive understanding of the compounds responsible for the observed effects and their potential molecular targets.

5 | Conclusion

The results of this study provide evidence that the PS-FS has significant potential as a dietary agent in the management of IBD. By modulating the Nrf2-antioxidant enzyme axis and enhancing the gene expression of TJ proteins, PS-FS addresses two critical aspects of IBD pathogenesis: oxidative stress and intestinal barrier dysfunction. These findings are particularly relevant in light of the ongoing search for treatments that are not only effective, but also have a favorable safety profile, given the chronic nature of IBD treatment. The ability of PS-FS to modulate key inflammatory and oxidative pathways suggests that it may represent a dietary strategy for management of intestinal inflammation. However, the antagonism observed with budesonide indicates that further research is needed to understand the interactions between PS and existing IBD medications. This could involve exploring different dosing strategies or identifying which anti-inflammatory drugs PS-FS can be coadministered with in order to enhance the beneficial effects of such combinations.

Author Contributions

Mussa Makran: investigation, writing – original draft, formal analysis, data curation. **Ilenia Concetta Giardina**: investigation, writing – original draft, formal analysis, data curation. **Guadalupe Garcia-llatas**: conceptualization, funding acquisition, writing – review and editing, project administration, supervision. **Alessandro Attanzio**: conceptualization, funding acquisition, writing – review and editing, project administration, supervision. **Antonio Cilla**: supervision, project administration, writing – review and editing, funding acquisition, conceptualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data generated during the current study are available from the corresponding authors upon reasonable request.

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