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Pigmented corn as a gluten-free source of polyphenols with anti-inflammatory and antioxidant properties in CaCo-2 cells

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ABSTRACT

A high number of varieties from corn (*Zea mays* L.) have been consumed for long time all over the world, however pigmented varieties are recently gaining renewed attention due to their beneficial effects and polyphenolic content.

The natural lack of gluten makes corn suitable for consumption by celiac population, who need to control their inflammatory state through an appropriate gluten-free diet. The biological effects of polyphenols from pigmented corn are poorly investigated in the context of celiac disease. In this work, we analyzed through HPLC-DAD the phenolic composition of two Italian purple and red varieties ("Scagliolo Rosso" and "Rostrato di Rovetta", respectively) comparing their effects in human intestinal epithelial cells (CaCo-2 cells). The possible impact of gastro-intestinal digestion following oral consumption was assessed as well.

The phenolic profile showed the presence of phenolic acids in both varieties, while anthocyanins were identified in Scagliolo Rosso only. After simulated digestion, the level of polyphenols did not significantly change and paralleled with an increased scavenging activity. In CaCo-2 cells, stimulated by a proinflammatory cocktail containing gliadin-derived peptides (IL-1β, IFN-γ, digested gliadin), pigmented corn extracts inhibited the release of CXCL-10 and sICAM-1, with mechanisms partially ascribed to NF-κB impairment. At the same concentration (200 μg/mL), ROS production and catalase depletion were reverted through Nrf-2-independent mechanisms.

Our data suggest that polyphenols from pigmented corns might help in controlling the inflammatory and oxidative state of people with celiac disease at intestinal level, at concentrations potentially achievable through a gluten-free diet.

1. Introduction

Corn (*Zea mays* L.) has a long-standing history of consumption beginning with the use by indigenous Americans as typical food already more than four thousand years ago. Today, corn remains an essential food in the diet of different cultures in Central and South America, but also plays an important role in human nutrition and animal feeding all over the world. Several varieties of corn, including those with pigmented seeds such as black, red, and yellow, were already known by native Americans (Werner, 1997).

Based on this long traditional use as food, several varieties have been selected over the years by traditional breeding methods or developed by genetic modification to improve their nutritional quality (see the review from Petroni *et al.* (Petroni et al., 2014)). Beyond the macronutrients, the abundance of secondary metabolites, mostly polyphenols, is relatively poor in the widely used varieties of yellow and white corn. Typically, they contain about 0.1 % of total phenols, mostly represented by phenolic acids, such as *p*-coumaric and ferulic acids (Cuevas Montilla et al., 2011; Syedd-Leon et al., 2020). Several groups, including ours, evaluated the phenolic profile of pigmented corns in comparison to

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common yellow varieties: the first showed a higher amount and heterogeneity of polyphenols, among which anthocyanins are considered mostly responsible for several biological activities, including improvement of oxidative stress (Bani et al., 2023; F. Colombo et al., 2021; Cuevas Montilla et al., 2011; Suriano et al., 2021; Syedd-Leon et al., 2020). In general, pigmented cereals have reached a renewed attention as food ingredients useful to supply a significant amount of polyphenols in human diet, thus providing potential benefits against cardiometabolic risk among others (please see reviews from Mbanjo and Colombo (R. Colombo et al., 2021; Mbanjo et al., 2020)).

Due to the natural lack of gluten, corn is one of the cereals traditionally included in a gluten-free diet, which is necessary to prevent the intestinal damage in patients with celiac disease (CeD). However, residual oxidative and inflammatory markers may persist in non-responders to gluten-free diet (Manavalan et al., 2010; Moretti et al., 2018; Odetti et al., 1998). The pathogenesis of CeD is classified as an autoimmune response, orchestrated by gluten-specific T cells releasing IL-21 and IFN-γ (for a deeper reading on the molecular hallmarks of CeD, see the exhaustive review from Ramírez-Sánchez *et al.* (Ramirez-Sanchez et al., 2020). In CeD, small peptides produced from incomplete digestion of gliadin may cross the intestinal barrier eliciting the inflammatory response, ultimately resulting in epithelial atrophy. In addition to the well-known activation of STAT-1 pathway, IFN-γ co-operates with effector cytokines, such as IL-1β and TNF-α, playing a key role in the activation of other inflammatory transcription factors, including NF-κB (Van De Walle et al., 2010).

The role of pigmented cereals devoid of gliadin for the control of the oxidative and inflammatory status of celiac population is poorly investigated. By previous in vitro experiments, we suggested that Italian varieties of pigmented corn and rice might represent relevant dietary source of phenolic acids and anthocyanins (Bani et al., 2023; F.Colombo et al., 2021; Piazza et al., 2022). Moreover, we also suggested that extracts from black rice might contribute to reduce the inflammation and oxidative stress caused by cytokines and gliadin at intestinal level, by acting on the NF-κB pathway and ROS production at concentrations of 50 – 200 μg/mL (Piazza et al., 2022). Similarly, in the present work, we evaluate the composition, the stability, and the biological effect of polar extracts from two purple and red varieties of corn ("Scagliolo Rosso" and "Rostrato di Rovetta", respectively), in an in vitro model of intestinal inflammation typical of CeD, using human intestinal epithelial CaCo-2 cells stimulated with pro-inflammatory cytokines (IL-1β, IFN-γ) in the presence of digested gliadin or pro-oxidant damage (H_2O_2).

2. Materials and methods

2.1. Materials

Methanol, ethanol, HPLC-grade water, acetonitrile, acetone, toluene, hydrochloric acid, reagents for electrophoretic analysis were from VWR International (Fontenay-sous-Bois, France). Gliadin, digestive enzymes (pepsin, trypsin), Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), 2,2′-Azino-bis(3– ethylbenzothiazoline – 6 – sulfonic acid) diammonium salt (ABTS), 2-Aminoethyl diphenylborinate (NP), MG-132, sodium carbonate, sodium butyrate, phenolic acids (coumaric acid, ferulic acid, chlorogenic acid, vanillic acid, gallic acid, syringic acid, dihydroxybenzoic acid), rutin, kaempferol, quercitin, and quercetin-3-O-glucoside were purchased from Sigma Aldrich (Merck group, Steinheim, Germany).

Apigenin was purchased from Phytolab (Dutendorfer Straße 5–7, 91,487 Vestenbergsgreuth, Germany). Anthocyanin standards (cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside) were from Extrasynthese (Genay, France). Pre-stained molecular weight marker solution was from Bio-Rad (Hercules, CA, USA).

All the disposable materials were from Euroclone (Pero, Italy) and Corning (Corning Life Sciences, Amsterdam, The Netherlands). Cell media (DMEM) and supplements (sodium pyruvate, non-essential ammino acids) were from Sigma Aldrich, while trypsin-EDTA, glutamine, and pen./strep. mix of antibiotics were from GibcoTM (Thermo Fisher Scientific, Monza, Italy). FBS was from Euroclone. Human cytokines and Human ELISA ABTS kits were from PeproTech (London, UK).

2.2. Corn samples and extraction method

Corn samples (seeds from *Zea mays* L.) belonging to the variety "Rostrato di Rovetta" (RR) was supplied by the Italian farm "Associazione Rosso Mais" (Rovetta, BG, Italy). RR corn samples were cultivated in Northern Italy in the hilly area of Rovetta, Fino del Monte, Songavazzo and Cerete (Bergamo, Italy). In 2016, the variety was registered in the National Register of Conservation Varieties (GU n.251 del 26–10-2016). RR samples were sowed in May 2021 and harvested in October 2021. "Scagliolo Rosso" (SR) was produced in the experimental field of the University of Milan located in Landriano (PV, Italy). More specifically, "Scagliolo Rosso" was obtained through five recurrent backcrosses between a purple synthetic variety carrying the B1 and Pl1 anthocyanin regulatory genes and the yellow "Scagliolo variety" [2]. Plants with the highest anthocyanin content in seeds then underwent more than three cycles of self-pollination. Corn seeds from "Scagliolo Rosso" used in this study were obtained from plants grown and selfed in the 2019 field season and then harvested at the same time at the end of the season. Corn samples were grinded using a coffee mill and extracted according to our previous paper (Piazza et al., 2022). Each corn flour (20 g) was extracted twice with 200 mL of ethanol:water 60:40 (v/v) for 4 and 16 h, respectively, under dark conditions at room temperature. The mixtures obtained were filtered through filter paper with particle retention of 10–20 μm (VWR, France), concentrated using a Rotavapor (Heidolph Instruments GmbH & CO, Schwabach, Germany), freeze-dried (Edwards, 5Pascal, Trezzano, Italy), and kept at − 20 ∘C. The powder was dissolved by water:DMSO (50:50) mixture, aliquoted and kept at − 20 ∘C for the biological assays. Recovery % (w/w) was 6.23 and 7.5 for Scagliolo Rosso and Rostrato di Rovetta, respectively.

2.3. Peptic/Tryptic in vitro digestion

To reproduce an inflammatory condition mimicking the celiac disease at the gut level, the gliadin and the corn extracts were in vitro digested following a peptic/tryptic protocol. The more complex gastro-intestinal in vitro digestion reported by Sangiovanni *et al.* (Sangiovanni et al., 2015) and the peptic/tryptic protocol was applied on a hydro-alcoholic phenolic extract showed similar effect on the phenolic profile (data not shown); therefore, the simplified protocol of Van Buiten *et al.* (Van Buiten et al., 2018) was selected in this study to reduce interference on in vitro cellular assays.

The in vitro digestion was performed as described by Van Buiten *et al.* with minor modifications; briefly, 2 g of gliadin or 0.1 g of corn extracts were suspended in 20 mL of 0.2 N HCl and stirred at 37 °C. After 10 min, pepsin (rate 1:50 enzyme:substrate w/w) was added, and the solutions were stirred for 2 h at 37 ∘C. At the end of the gastric digestion, the pH was adjusted to 7.4 using 2 N NaOH, trypsin (rate 1:50 enzyme: substrate w/w) and the solutions were stirred for 4 h at 37 ∘C. After the gastro-intestinal digestion, the solutions obtained were freeze-dried and maintained at − 20 °C until the assays. A blank, containing only the digestive enzymes, was prepared at the same time to evaluate possible interference during the assays.

The gliadin digestion was monitored using the Sodium Dodecyl Sulphate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE) technique, according to the method by Ballabio *et al.* (Ballabio et al., 2011). Digested gliadin was suspended in sample buffer (0.125 M Tris-HCl, pH 6.8, 3.75 % glycerol, 1 % SDS, 5 % β-mercaptoethanol) diluted with water (1:1, v/v) on a polyacrylamide gradient gel (9–19 % acrylamide). After the electrophoretic run (90 V at room temperature, for 6 h), gels were maintained for 20 min in 20 % TCA at 4 °C and stained with Coomassie Brilliant Blue G-250.

2.4. Analysis of the phenolic profile and antioxidant capacity

2.4.1. Determination of total anthocyanin content

2.4.1.1. pH differential assay. The total anthocyanin content in the pigmented corn samples was determined spectrophotometrically, as described by the AOAC method (Horwitz and Latimer, 2005).

A total of 25 mg of each extract was weighed, dissolved in 5 mL of methanol:1 M hydrochloric acid solution (85:15), and filtered through a 0.45 μm PTFE filter (VWR International, Fontenay-sous-Bois, France).

The absorbance of the samples, suitably diluted with buffer solutions at pH 1.0 (0.025 M potassium chloride) and pH 4.5 (0.4 M sodium acetate), was measured at 520 nm and 700 nm, with the second reading used to eliminate interference.

The total anthocyanin content (TA) is expressed as equivalents of cyanidin-3-O-glucoside (CY mg/g), following the relationship:

TA (CY mg/g) = ΔA x MW x DF x 1000 x V/e x l x W.

Where: ΔA is the difference between (A520nm-A700nm) at pH 1.0 and (A520nm-A700nm) at pH 4.5; MW is the molecular weight (449.2 g/mol for cyanidin-3-O-glucoside); DF is the dilution factor; 1000 is the conversion factor from g to mg; V is the extraction volume (L); e is the molar extinction coefficient (26,900 for cyanidin-3-O-glucoside); l is the optical path length expressed in cm (1 cm); and W is the sample weight (g).

2.4.1.2. Quantification of anthocyanins using HPLC-DAD. The total anthocyanin content was evaluated before and after digestion using the HPLC-DAD technique with the method published by Colombo and collaborators (2021) (F. Colombo et al., 2021). A total of 25 mg of each extract was weighed, dissolved in 5 mL of methanol and 1 M hydrochloric acid solution (85:15), and filtered through a 0.45 μm PTFE filter (VWR International, Fontenay-sous-Bois, France). When necessary, appropriate dilutions were made using 0.1 N HCl.

Briefly, the analysis was conducted utilizing a Jasco HPLC equipment (Jasco, Tokyo, Japan) equipped with a pump (PU-980), an interface (LC-NETII/ADC), a diode array detector (MD-2010 Plus), a mixer (LG-150–0.4), a degasser (DG-2080–54), and an injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20 μL loop. A Synergy 4 μ MAX-RP 80A column (250 x 4.60 mm, 4 μm), along with a Security Guard C12 pre-column (4 x 3.0 mm ID) from Phenomenex, Torrance, CA, USA, was employed for separation. The flow rate was set at 0.8 mL/min^{-1}. The gradient was set up as follows: 0–15 min: 94 %–70 %, A, 15–30 min: 70 %–50 % A, 30–35 min: 50 %–10 % A, 35–38 min: 10 % An isocratic A; where (A) water:acetonitrile:formic acid 96:3:1 (v/v/v); and (B) acetonitrile:water:formic acid 50:49:1 (v/v/v). The anthocyanins were detected at 520 nm. The stock solutions of cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, and peonidin-3-O-glucoside were prepared by dissolving 2 mg of each standard anthocyanin in 10 mL of 0.1 N HCl to achieve a final concentration of 200 μg/mL. Calibration curves were constructed for each standard: cyanidin 3-O-glucoside: concentration range 0.5——20 μg/mL; pelargonidin 3-O-glucoside and peonidin 3-O-glucoside: concentration range 0.5——10 μg/mL.

2.4.2. Determination of soluble phenolic content

Phenolic compounds are found in cereals, both in soluble form and bound to cell wall components. In this study, we focused on the free phenolic compounds because they are readily available for absorption in the small human intestine (see the suggested review from Manach *et al.* for the topic (Manach et al., 2004)).

2.4.2.1. Folin–Ciocalteu's assay. The Folin–Ciocalteu assay, in accordance with the method reported by Singleton and Rossi (1965) (Singleton, 1965), was employed to determine the Total Phenolic Content (TPC) of extracts. Initially, the extracts, both before and after digestion, were dissolved in a 60:40 (v/v) mixture of ethanol and water at a final concentration of 2 mg extracts per mL. Subsequently, all samples were sonicated for 5 min, and they were filtered using a 0.45 μm filter (VWR International, Fontenay-sous-Boys, France). To establish a calibration curve, gallic acid standard solutions ranging from 5 to 50 μg/mL were prepared. For the analysis, 300 μL of diluted samples or standards were combined with 1.5 mL of 0.2 N Folin–Ciocalteau reagent and 1.2 mL of a 7.5 % sodium carbonate solution. To eliminate interference, a digestive blank and water were concurrently analyzed. The solutions were incubated in the dark for 30 min, after which their absorbance was measured at 765 nm using a UV–visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, CA, USA). The results were quantified in terms of mg/g equivalent of gallic acid (GAE).

2.4.3. Determination of in vitro antioxidant capacity

The antioxidant capacity of corn extracts was assessed through spectrophotometric methods, specifically the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and the Trolox Equivalent Antioxidant Capacity (TEAC) test. Both the pre-digestion and post-digestion extracts were prepared by dissolving them in a solution of ethanol and water (60:40, v/v) to achieve a final concentration of 2 mg extracts per mL. These solutions were sonicated for 5 min and then filtered through 0.45 μm filter (VWR International, Fontenay-sous-Boys, France).

2.4.3.1. DPPH assay. In the DPPH assay (Brand-Williams et al., 1995, (Brand-Williams, 1995)), 1 mL of a 0.005 % DPPH solution in methanol was added to the samples or a blank (0.5 mL). This mixture was kept in the dark for 30 min. Subsequently, the absorbance was measured against a methanol reference at 517 nm. To calculate the antioxidant capacity, a calibration curve was constructed by plotting the concentration of gallic acid (ranging from 1.0 to 5.0 μg/mL) against the difference between the absorbance of the blank and the absorbance of standards (A0-A). The results were expressed in terms of mg/g equivalents of gallic acid (GAE).

2.4.3.2. TEAC assay. The Trolox Equivalent Antioxidant Capacity (TEAC) assay, following the method by Re et al., 1999 (Re et al., 1999) with some modifications, involved preparing an ABTS radical cation solution. This solution was created by mixing 2.45 mM of potassium persulfate with 7 mM of ABTS (in a 1:1 v/v ratio). The resulting mixture was left at room temperature in the dark for 12–16 h. Before use, the ABTS radical solution was diluted with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm.

For the assay, 150 μL of each sample or blank was mixed with aliquots of 1.5 mL of the ABTS+• solution, and after 6 min, the absorbance was measured at 734 nm. The antioxidant capacity was expressed as mg/g of Trolox equivalents (TE) and was calculated using a calibration curve generated by plotting the concentration of trolox (ranging from 10 to 30 μg/mL) against the percentage of inhibition of ABTS+•, which was calculated using the formula:

$$\%Inhibition of ABTS+\bullet = [(Ab - At)/Ab] \times 100$$

where: Ab: absorbance of the blank. At: absorbance of the test solutions.

2.4.3.3. High Performance Thin Layer Chromatography. The evaluation of the samples' fingerprints and simultaneous testing of their antioxidant properties were facilitated by High-Performance Thin Layer Chromatography (HPTLC). This method allowed for the assessment of sample characteristics and antioxidant properties through HPTLC, following the procedure established by Colombo et al., 2021 (F. Colombo et al., 2021).

In brief, for each analysis, 15 μL of corn extracts (prepared at a concentration of 8 mg extracts per mL in ethanol:water 60:40 v/v) or digestion blank and aliquots of 5 μL of standard solutions (coumaric acid, ferulic acid, chlorogenic acid, vanillic acid, gallic acid, syringic acid, dihydroxybenzoic acid, rutin, kaempferol, quercetin, and quercetin-3O-glucoside) at the concentration of 200 μg/mL were loaded

onto HPTLC silica-gel plates 60 F254 (dimensions: 10 × 20 cm, manufacturer: Merck, Darmstadt, Germany). This was accomplished using a semi-automatic sample applicator (Linomat 4, CAMAG, Muttenz, Switzerland).

In order to evaluate the antioxidant activity associated with each compound present in the samples, HPTLC technique was applied as follows: after the chromatographic run (mobile phase: 10 mL of acetone: toluene:formic acid 4.5:4.5:1 *v/v/v*), the plates were revealed at 366 nm, derivatized with 0.05 % DPPH methanolic solution, maintained for 30 min in the dark and revealed at visible light (software VisionCats, CAMAG, Muttenz, Switzerland).

On the other hand, in order to evaluate the content of flavonoids, anthocyanins and acids present in the samples, after the chromatographic run (mobile phase: ethyl acetate:acetic acid:formic acid:H_2O 10:1.1:1.1:2 *v/v/v/v*), the plates were revealed at 366 nm. Then, the plates were derivatized using a 1 % 2-Aminoethyl diphenylborinate (NP) methanolic solution, kept in the dark for 30 min, and examined at 366 nm using VisionCats software (CAMAG, Muttenz, Switzerland).

2.5. In vitro evaluation of biological activity

2.5.1. Cell culture and treatment

Human epithelial cells from colorectal cancer, CaCo-2 (clone HB237, ATCC, Manassas, VA, USA) were cultured with high glucose DMEM supplemented with 100 mg/mL streptomycin, 100 units/mL of penicillin, 1 % non-essential amino acids, 1 mM sodium pyruvate, 4 mM L-glutamine and 10 % FBS (Fetal Bovine Serum). Cells (5 x 10^5) were subcultured before reaching confluency using Trypsin-EDTA 0.25 %, counted by Bürker chamber, and seeded in 75 cm^2 flasks for 48–72 h; then, cells were incubated under humidified atmosphere with 5 % CO_2 at 37 °C. Similarly, for each biological experiment, cells were counted and seeded in different plates for 48 h using supplemented DMEM before treatment.

For inflammation experiments, cells were stimulated with a pro-inflammatory cocktail composed by IL-1β (10 ng/mL), IFN-γ (10 ng/mL), and in vitro digested gliadin (Ga) (1 mg/mL). For oxidative stress experiments, cells were challenged by hydrogen peroxide (H_2O_2) 1 mM. For all treatments, DMEM without FBS supplementation was used. The selected read-outs were the NF-κB- and STAT-1-driven transcription (6 h), the release of inflammatory mediators (24 h), and the production of intracellular ROS (1 h).

2.5.2. Measurement of cell viability

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured by the 3,4,5-dimethylthiazol- 2-yl-2–5-diphenylte-trazolium bromide (MTT) method after 6 h and 24 h (Fig. S1). The MTT solution (Phosphate Buffer Solution, PBS 1X, 200 μg/mL) was added to cell after removing cell media at the end of the biological treatment with corn extracts. The resulting formazan salts (purple) were dissolved by isopropanol:DMSO (90:10 v/v) solution after 15–30 min of metabolization. Finally, the absorbance was read at 550 nm and directly correlated with cell viability.

2.5.3. ELISA assays on inflammatory mediators

Cells were seeded in 24-well plates (3 x 10^4/well) for 48 h, then treated with the pro-inflammatory cocktail (IL-1β, IFN-γ, Ga) and corn extracts (RR, SR) for 6 h. The release of human CXCL-8 (IL-8), CXCL-10, and sICAM-1 (soluble form of ICAM-1) in CaCo-2 cell media were measured by ELISA development ABTS kits according to the manufacturer's instructions (PeproTech, London, UK), as previously reported (Piazza et al., 2022). Corning 96-well EIA/RIA plates (Merck Life Science, Milano, Italy) were coated with the capture antibodies and kept at room temperature overnight. The amount of inflammatory mediators in the culture media was detected by the measurement of the absorbance resulting from the colorimetric reaction between ABTS substrate (Merck Life Science, Milan, Italy) and horseradish peroxidase enzyme. The absorbance was read using a spectrophotometer (VICTOR X3; PerkinElmer, Milano, Italy) at 405 nm (0.1 sec.). The results (pg/mL) were expressed as percentage versus the stimulated control, which was arbitrarily assigned the value of 100 %. Apigenin (20 μM) was chosen as well-known anti-inflammatory and antioxidant polyphenol, according to previously published papers (Hoensch & Weigmann, 2018).

2.5.4. Transcription factors driven transcription

Cells were seeded in 24-well plates (3 x 10^4/well) for 48 h, then transfected with plasmids containing elements responsive to κB (100 ng per well), Nrf-2 (200 ng per well), or STAT-1 (100 ng per well). The activity of both transcription factors was evaluated after 6 h of treatment with corn extracts. NF-κB plasmid was a gift from Dr. N. Marx (Department of Internal Medicine-Cardiology, University of Ulm; Ulm, Germany), while Nrf-2 and STAT-1 was shared by O'Connell *et al.* (O'Connell et al., 2016) through the Addgene non-profit repository (#90398, #90338, Addgene, LGC Standards, Teddington, UK). As previously reported (Piazza et al., 2022), CaCo-2 cells were transiently transfected by Lipofectamine® 3000 Reagent (Invitrogen®; Thermo Fisher Scientific, Monza, Italy), following manufacturer instructions. Britelite™ Plus reagent (Perkin Elmer, Milano, Italy), containing luciferin, was used to assess the amount of luciferase produced into the cells at the end of the treatment. A plate reader (VICTOR X3, Perkin Elmer) was used to measure luminescence deriving by the reaction between luciferin and luciferase. Apigenin 20 μM was used as reference antioxidant and anti-inflammatory compound, able to modulate either NF-κB and Nrf-2 pathways; the proteasomal inhibitor MG-132 1 μM was used as well-known activator of Nrf-2 translocation (Geillinger et al., 2014; Svehlikova et al., 2004).

2.5.5. Intracellular production of ROS

CaCo-2 cells (10^4/well) were cultured in black 96-well plates for 48 h. Then, corn extracts (RR, SR) were added for 24 h to allow the potential induction of endogenous antioxidant defense. The redox sensitive probe CM-H2DCFDA (InvitrogenTM, Thermo Fisher Scientific, Monza, Italy) 10 μM in PBS 1X solution was added for 30 min for cell uptake. Then, the excess of probe was removed by washing with PBS 1X and the oxidative stress was induced by H_2O_2 (1 mM) for 1 h, as previously conducted by many authors (Pahlke et al., 2021; Piazza et al., 2022). At the end, cells were washed with PBS 1X and 100 μL of PBS were added to each well to read the emitted signal. Fluorescence was read using a multiplate reader (Victor × 3; PerkinElmer, Milano, Italy) at ex. 490 nm/em. 535 nm.

2.5.6. Enzymatic activity of catalase

Cells were seeded in 24-well plates (3 x 10^4/well) for 48 h, then treated with corn extracts (RR, SR) for 24 h, followed by treatment with H_2O_2 (1 mM) for 1 h. As previously reported (Piazza et al., 2022), the recovery of catalase levels was indirectly evaluated by enzymatic assay, according to manufacturer instructions (Cayman chemical, Ann Arbor, MI, USA). Samples were obtained by lysis with cold phosphate buffer (KH_2PO_4 50 mM, EDTA 1 mM, Triton-X 0.1 %; pH 7), immediately at the end of the treatment. In brief, 20 μg of total proteins from intracellular lysate (generally corresponding to 20 μL of sample) were incubated with methanol (20 μL) to measure its colorimetric conversion into formaldehyde in the presence of H_2O_2 (peroxidase activity). The absorbance of samples at 535 nm was compared with that of formaldehyde calibration curve (0 – 75 μM) (Victor × 3; PerkinElmer, Milano, Italy).

2.5.7. Measurement of epithelial barrier integrity

CaCo-2 cells (3 x 10^5/well) were differentiated on Transwell® support for 17–21 days, as previously described (Cremonini et al., 2017). Cell medium was replaced every other day, by adding supplemented DMEM medium to the basolateral compartment and FBS-free medium to the apical compartment. The integrity of the epithelial monolayer was

measured by EVOM3 device (WPI, Sarasota, Florida, USA), as trans electrical resistance (*TEER*, Ω), with cut-off value above 400 Ω.

The basolateral compartment of differentiated cells was treated with the pro-inflammatory cocktail (IL-1β, IFN-γ, Ga) to simulate the endogenous inflammation triggered by gliadin, while corn extracts (RR, SR) were added to the apical compartment to simulate the luminal exposure through diet. TEER measurements were recorded before and after treatment (24 h), and the variation was calculated ($\Delta\Omega = \Omega t_{24h} - \Omega t_0$). Results were expressed as mean of ΔΩ for each treatment condition ± SEM. Sodium butyrate (2 mM) was used as well-established short chain fatty acid (SCFA) with protective effect on gut barrier integrity (Huang et al., 2021).

2.5.8. Statistical analysis

Comparison of analytical data between extracts before and after digestion was performed by parametric paired *t*-test using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). The correlation between variables was assessed by Pearson linear correlation using IBM SPSS Statistics, Version 27.0 (New York, NY, USA). All biological data were expressed as the mean ± SEM of at least three independent experiments. Unpaired one-way analysis of variance (ANOVA) was used to analyze the quantitative assays, followed by the Bonferroni post-hoc test using GraphPad Prism. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of extracts

3.1.1. Phenolic profile and antioxidant capacity

Different spectrophotometric methods were employed to chemically characterize the corn extracts. Table 1 reports the measurement of Total Phenolic Content (TPC) and Total Anthocyanin Content (TAC) in both pre- and post-digestion samples. Concurrently, Antioxidant Capacity (AOA) was assessed using DPPH and TEAC assays.

The TPC ranged between 63.63 ± 5.98 (Scagliolo Rosso) and 27.1 ± 1.40 (Rostrato di Rovetta). Scagliolo Rosso showed the highest total phenolic content before and after digestion compared to the Rostrato di Rovetta extracts. In all the samples analyzed, the content of soluble polyphenols did not vary significantly before and after the in vitro digestion ($p > 0.05$), while Scagliolo Rosso showed higher TPC after digestion (63.63 ± 5.98 vs 50.75 ± 6.36).

The TAC was quantified in one corn extract sample (7.16 ± 0.42 (before digestion), 6.62 ± 0.17 (after digestion) mg cyanidin-3-O-glucoside equivalents (CY)/g). Scagliolo Rosso extracts showed the highest phenolic content and were rich in anthocyanins whereas this class of molecules was not detected in Rostrato di Rovetta.

The AOA ranged between 9.20 ± 0.50 (Scagliolo Rosso) and 3.62 ± 0.21 (Rostrato di Rovetta) for DPPH assay and ranged between 109.81 ± 3.66 (Scagliolo Rosso) and 51.41 ± 2.98 (Rostrato di Rovetta) for TEAC assay.

Table 1
Evaluation of the phenolic composition of corn extracts from pigmented varieties "Scagliolo Rosso" and "Rostrato di Rovetta".

Sample	Digestion	TPC mg GAE/g	TAC mg CY/g	AOA	
				DPPH mg GAE/g	**TEAC mg TE/g**
Scagliolo Rosso	Before	50.75 ± 6.36	7.16 ± 0.42	8.33 ± 0.58	73.86 ± 5.30
	After	63.63 ± 5.98	6.62 ± 0.17	9.20 ± 0.50	109.81 ± 3.66*
Rostrato di Rovetta	Before	28.42 ± 1.63	–	3.62 ± 0.21	51.41 ± 2.98
	After	27.10 ± 1.40	–	4.15 ± 0.23*	59.74 ± 7.59

The results (mean ± SD, n = 3) are expressed as mg equivalent of gallic acid (GAE)/g, mg equivalent of cyanidin-3-O-glucoside (CY)/g, or mg Trolox equivalent (TE)/g ± standard deviation. TPC: Total Phenolic Content; TAC: Total Anthocyanin Content; AOA; Antioxidant Capacity; *$p < 0.05$.

3.1.2. Identification and quantification of phenolic compounds by chromatographic techniques

To investigate the differences between corn samples during gastrointestinal digestion, High-Performance Thin Layer Chromatography (HPTLC) technique was employed. HPTLC is a semi-quantitative, rapid, and suitable method for assessing the phenolic profile of samples. Furthermore, this technique can be utilized to assess the antioxidant activity associated with each compound present in the samples. Fig. 1 depicts the HPTLC separation of samples before and after in vitro digestion. The determination of the antioxidant activity of the main compounds present in the samples was performed by derivatizing the plates with a DPPH (2.2-Diphenyl-1-picrylhydrazyl) solution (Fig. 1A). The detection of flavonoids, anthocyanins and plant acids in the samples was performed by derivatizing the plates with a NP (2-Aminoethyl diphenylborinate) solution followed by observation under UV 366 nm lamp (Fig. 1B). The characterization was carried out by comparing the chromatographic run of the standards with that of the samples, comparing the type and intensity of the band discoloration.

This method was useful to evaluate the antioxidant capacity of the specific compounds separated by chromatography, in contrast to

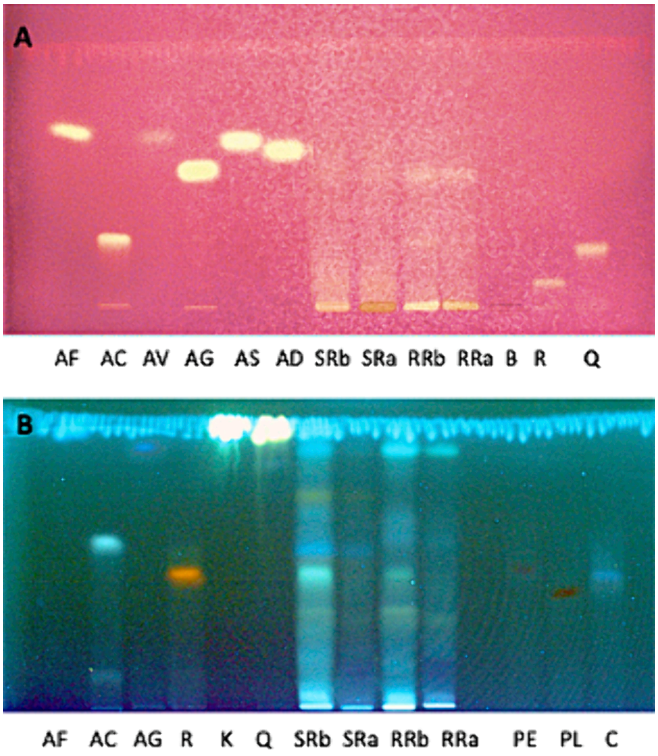

Fig. 1. A. HPTLC plate of standards and samples detected at visible light after derivatization with DPPH. AF: Ferulic acid, AC: Chlorogenic acid, AV: Vanillic acid, AG: Gallic acid, AS: Syringic acid, AD: dihydroxybenzoic acid, SRb: Scagliolo Rosso before digestion, SRa: Scagliolo Rosso after digestion; RRb: Rostrato di Rovetta before digestion, RRa: Rostrato di Rovetta after digestion, B: Blank of digestion, R: Rutin, Q: Quercetin-3-O-glucoside. **B.** HPTLC plate of standards and samples detected at 366 nm after derivatization with NP. AF: Ferulic acid, AC: Chlorogenic acid, AG: Gallic acid, R: Rutin, K: Kaempferol Q: Quercetin SRb: Scagliolo Rosso before digestion, SRa: Scagliolo Rosso after digestion; RRb: Rostrato di Rovetta before digestion, RRa: Rostrato di Rovetta after digestion, PE: Peonidin-3-*O*-glucoside, PL: Pelargonidin-3-*O*-glucoside, C: Cianidin-3-*O*-glucoside.

spectrophotometric methods that provide information about the total antioxidant capacity. All corn samples showed a complex fingerprint. In Fig. 1A, Rostrato di Rovetta variety, a band attributable to gallic acid (*Rf* = 0.45) was visible. However, this method could not take completely into account the contribution of anthocyanins to the AOA (due to the dark color of these molecules). The phenolic profile of all samples changed after in vitro digestion, and it was also characterized by a high antioxidant capacity against DPPH after digestion.

Accordingly, changes in phenolic composition after in vitro digestion were also observed by UV detection (Fig. 1B). Notably, in Scagliolo Rosso extract, the bands corresponding to chlorogenic acid (*Rf* = 0.71), rutin (*Rf* = 0.56) and peonidin-3-*O*-glucoside (*Rf* = 0.61) decreased after digestion. In Rostrato di Rovetta extract, the absence of anthocyanins and the presence of phenolic acids (light blue bands) was noticed.

As a second step, the HPLC-DAD method was used for the identification and quantification of the anthocyanins contained in the Scagliolo Rosso samples. The analysis was performed on both the pre-digested and post-digested samples.

As it can be observed from Fig. 2, six peaks were detectable: the first three were related to the anthocyanins-3-O-glucosides (identified through the reference standards) and the last three to the anthocyanins-3,6-malonylglucosides, identified by mass spectrometric analysis, as reported in our previous paper (F. Colombo et al., 2021). A reduction in anthocyanins was detected in Scagliolo Rosso corn sample before and after digestion. As the standards were not commercially available, the 3,6-malonylglucoside anthocyanins were quantified using the calibration curves of the corresponding 3-O-glucoside anthocyanins.

The identification and quantification of anthocyanins in Scagliolo Rosso corn extract before and after digestion by the HPLC-DAD method was listed in Table 2.

Table 2
HPLC-DAD analysis of anthocyanins in the pigmented variety "Scagliolo Rosso" before and after simulated digestion.

Compounds	Before-digestion (mg/g)	After-digestion (mg/g)	Reduction %
cyanidin-3-*O*-glucoside	1.74 ± 0.04	1.46 ± 0.03	−16.06
pelargonidin-3-O-glucoside	0.28 ± 0.00	0.21 ± 0.03	−23.76
peonidin-3-O-glucoside	0.79 ± 0.01	0.59 ± 0.06	−24.57
cyanidin-3,6-malonilglucoside	1.25 ± 0.01	0.88 ± 0.05	−29.61
pelargonidin-3,6-malonilglucoside	0.25 ± 0.00	0.17 ± 0.02	−33.02
peonidin-3,6-malonilglucoside	0.56 ± 0.01	0.41 ± 0.03	−26.63
Total	**4.87 ± 0.07**	**3.73 ± 0.20**	**−23.45**

The results are expressed as both average mg/g ± standard deviation of dry extract, and percentage (%) of reduction.

3.2. Evaluation of the biological activity in CaCo-2 cells

3.2.1. Effect of pigmented corn extracts on inflammatory mediators

Results from the previous analysis (Tables 1 and 2) suggested that the level of polyphenols was maintained after intestinal digestion of corn extracts. Thus, following experiments were carried out to investigate a possible parallelism among this observation and the biological activity.

First, the interference with cell viability was excluded by MTT test after 24 h of treatment with extracts under study (Fig. S1); then, well-known markers of tissue inflammation produced by human intestinal CaCo-2 cells, such as the chemokines CXCL-8/IL-8, CXCL-10, and the adhesion molecule ICAM-1 were measured before and after treatment with corn extracts. Of note, all selected mediators are known to be expressed through NF-κB- and STAT-related pathways in autoimmune diseases.

Several authors, including our group, showed that in vitro digested gliadin can be properly used to promote inflammation in CaCo-2 cells (Capozzi et al., 2013; Herrera et al., 2021; Monguzzi et al., 2019; (Piazza et al., 2022)), regardless the indirect activation of the immune system; however, the molecular mechanism have been only partially explained. In this regard, we previously observed that digested gliadin (Ga, 1 mg/mL) significantly enhanced the release of CXCL-10 when combined with IL-1β and IFN-γ. However, in contrast to IL-1β, gliadin enhanced the release of CXCL-10 through NF-κB-independent mechanisms (Piazza et al., 2022).

Accordingly, biological experiments were performed by treating CaCo-2 cells with corn extracts, at concentrations ranging from 50 to 200 μg/mL, in the presence of a pro-inflammatory cocktail composed by IL-1β, IFN-γ, and Ga. The release of the pro-inflammatory mediators was measured before and after applying the protocol for in vitro simulated gastrointestinal digestion.

Both extracts showed a concentration-dependent inhibition of CXCL-10 release, with IC_{50} of 104.5, and 160.4 μg/mL for Scagliolo Rosso and Rostrato di Rovetta, respectively (Fig. 3A). After simulated digestion, the inhibitory activity of Scagliolo Rosso was moderately but not significantly impaired (IC_{50} = 166.0 μg/mL), while the activity of Rostrato di Rovetta was significantly enhanced (IC_{50} = 74.2 μg/mL) (Fig. 3B).

A similar trend was observed for Rostrato di Rovetta on the release of sICAM-1 (Fig. 3C, 3D) since the inhibitory activity was increased following the simulated digestion (IC_{50} = 120.4 μg/mL); on the

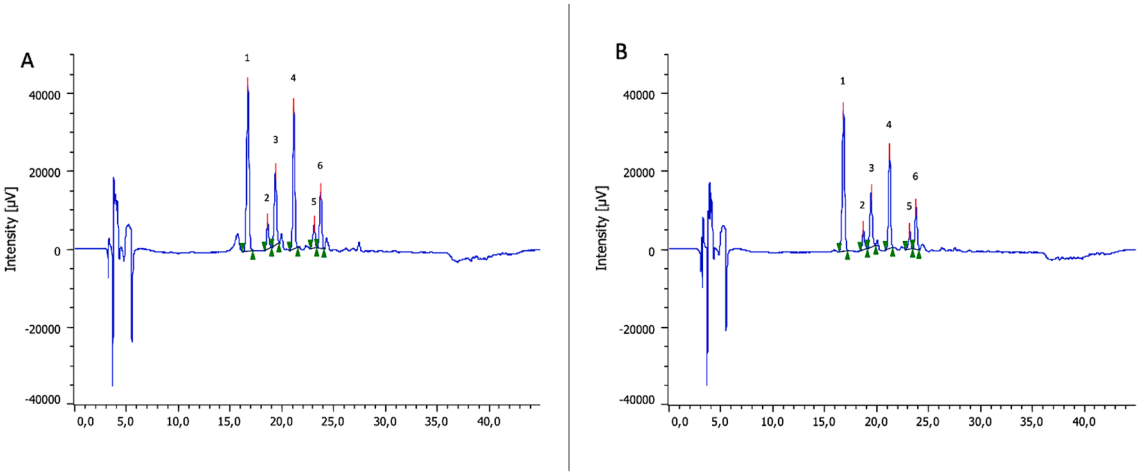

Fig. 2. Chromatograms of anthocyanins detected in Scagliolo rosso extract before **(A)** and after **(B)** in vitro digestion. 1) cyanidin-3-*O*-glucoside 2) pelargonidin-3-*O*-glucoside 3) peonidin-3-*O*-glucoside 4) cyanidin-3,6-malonilglucoside, 5) pelargonidin-3,6-malonilglucoside 6) peonidin-3,6-malonilglucoside.

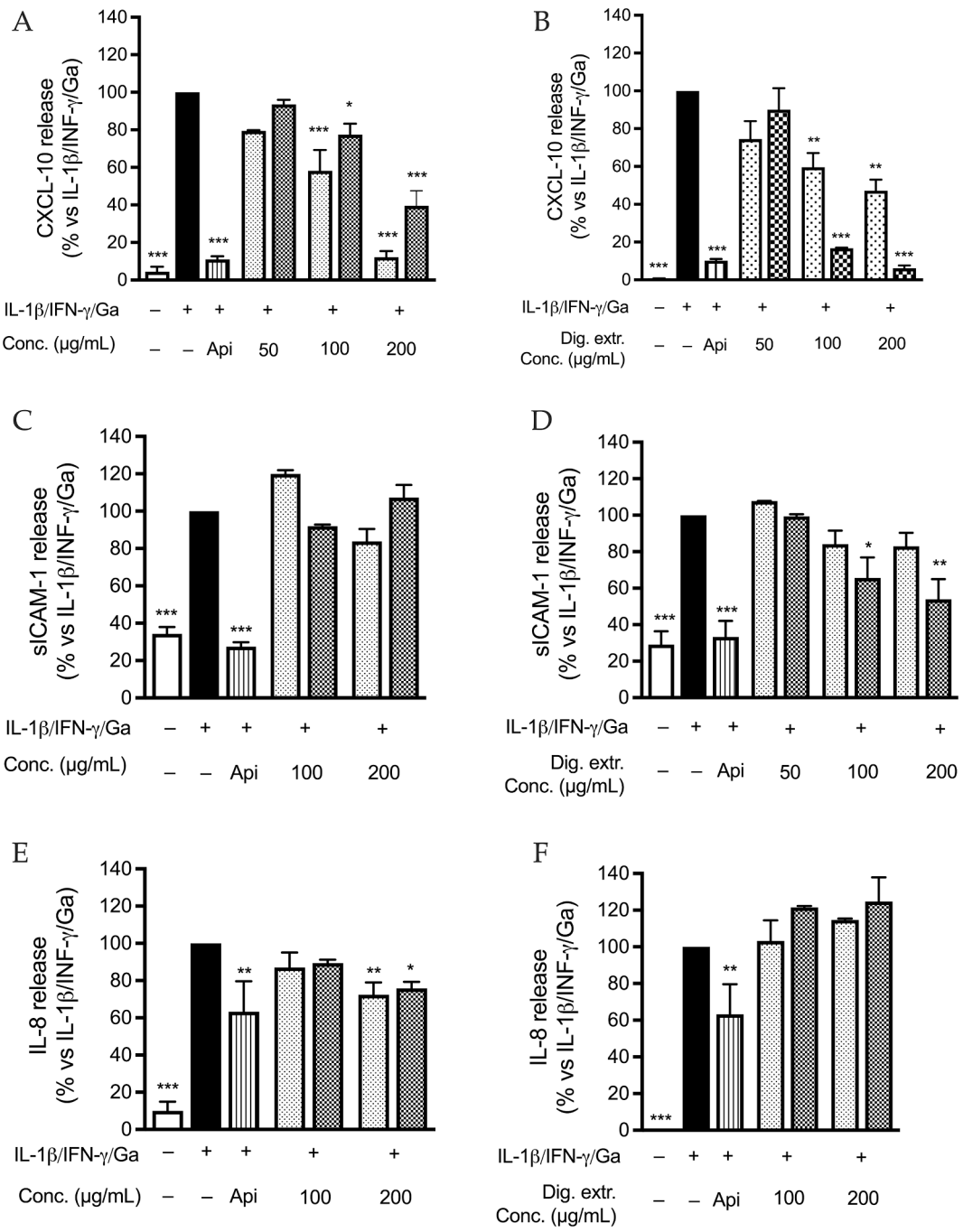

Fig. 3. Effect of pigmented corn extracts from varieties "Scagliolo Rosso" (dots) and "Rostrato di Rovetta" (squares) on the release of CXCL-10 (**A**, **B**), sICAM-1 (**C**, **D**), and IL-8 (**E**, **F**), measured by ELISA assay. Caco-2 cells were treated for 6 h with pro-inflammatory cocktail (black bar) and extracts, which evaluated before (**A**, **C**, **E**) and after simulated digestion (**B**, **D**, **F**). Apigenin 20 μM (vertical lines) was used as reference inhibitor. Data were expressed as mean (% of pg/mL) ± SEM relative to pro-inflammatory cocktail (stimulus), to which was arbitrary assigned the value of 100 %. Dig. extr., digested extracts; Ga, digested gliadin; Api, apigenin; *$p < 0.50$, **$p < 0.01$, ***$p < 0.001$, p values vs stimulus.

contrary, Scagliolo Rosso did not show any significant inhibition (−18 %) at the highest concentration tested.

The release of IL-8 was slightly reduced by both extracts (about −25 %) at 200 μg/mL (Fig. 3E), but the activity was lost after simulated digestion (Fig. 3F).

The experiments suggested that polyphenolic metabolites formed after intestinal digestion might interfere with molecular signals preferentially involved in the production of CXCL-10. For this reason, in addition to the key transcription factor NF-κB, the possible role of STAT-1 in our experimental conditions (IL-1β and IFN-γ signaling, respectively) was considered as well. Despite STAT-1 activation by cytokines has been clearly documented in the intestinal tissues of CeD patients, the direct involvement of gliadin-derived peptides in this pathway is still unclear, deserving further investigations.

Our data, reported in the supplementary materials (Fig. S2A), show that digested gliadin is a mild inducer of STAT-1 driven transcription in CaCo-2 cells, without any additional effect when combined with IL-1β and IFN-γ. Thus, STAT-1 activation might participate in the stimulatory effect of cytokines and digested gliadin on CXCL-10 release. Unfortunately, the treatment with corn extract at the highest concentration

tested (200 μg/mL) was not able to counteract the transcription driven by STAT-1 reporter plasmid (Fig. S2B).

On the contrary, the activity of NF-κB, stimulated by IL-1β, was moderately impaired by corn extracts at the same concentration (Fig. 4A), with negligible impact of the simulated digestion (Fig. 4B). The inhibitory activity was less pronounced, but still significant for Scagliolo Rosso, during the stimulation with the pro-inflammatory cocktail (Fig. 4C).

From one hand, these data confirm that NF-κB is involved, at least in part, in the bioactivity of corn extracts; on the other hand, our data support the hypothesis that additional pathways induced by the pro-inflammatory cocktail containing gliadin are modulated by Rostrato di Rovetta extract.

3.2.2. Effect of pigmented corn on oxidative stress

Our analytical data suggested that simulated digestion could positively impact on the antioxidant properties of corn extracts, measured by cell-free systems (Tab. 1). Thus, the first set of experiments has been carried out on oxidative stress mediators in human CaCo-2 cells, measuring the intracellular production of ROS, induced by H_2O_2. For this purpose, cells were treated for 24 h with corn extracts, before the oxidative injury (1 h), according to methods reported in the literature (Pahlke et al., 2021; Piazza et al., 2022). To resume the impact of intestinal digestion, we selected the concentration of 200 μg/mL from previous experiments, which activity was compared before and after simulated gastro-intestinal digestion.

Results showed in Fig. 5 demonstrate that both extracts significantly prevent ROS production (from −42 to −55 %), with similar activity to the reference antioxidant compound apigenin 20 μM (−68 %) and negligible difference after simulated digestion.

Consequently, we speculated about the molecular mechanisms behind the antioxidant effect of corn extracts. Since our experimental settings comprised a preventive treatment, we supposed that cell exposure to polyphenols could have enhanced the endogenous defense before the oxidative damage occurred. Accordingly, we decided to investigate the effect of corn extracts on the Nrf-2 pathway, in the absence of oxidative stress, as putative mechanism for the expression of antioxidant enzymes, such as catalase.

As shown in Fig. 6, corn extracts were unable to increase the activity of Nrf-2 at the concentration range of 50 – 200 μg/mL, contrarily to apigenin (20 μM) and proteasome inhibitor (MG-132, 1 μM) (Fig. 6A). In line with this observation, catalase levels were not increased at the highest concentration tested (data not shown). However, in the context of oxidative stress (i.e·H_2O_2), corn extracts preserved the activity of catalase at levels close to unstimulated control, although not significantly in respect to the stimulus (Fig. 6B). It was unexpected, even

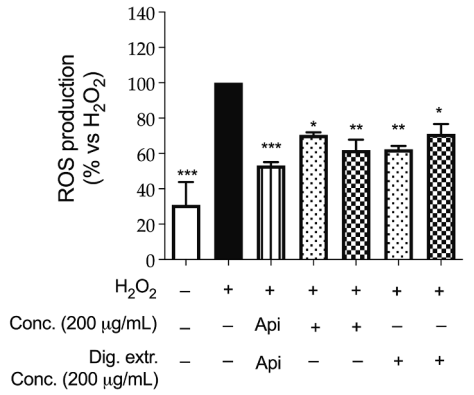

Fig. 5. Effect of pigmented corn extracts from varieties "Scagliolo Rosso" (dots) and "Rostrato di Rovetta" (squares) on intracellular ROS production, measured by DCF probe. Caco-2 cells were treated for 24 h with extracts followed by 1 h with H_2O_2 (black bar). Apigenin 20 μM (vertical lines) was used as reference inhibitor. Data were expressed as mean (% of emission units) ± SEM relative to H_2O_2 (stimulus), to which was arbitrary assigned the value of 100 %. Dig. extr., digested extracts; Api, apigenin; *p < 0.50, **p < 0.01, ***p < 0.001, p values vs stimulus.

though out from our focus, to note that reference Nrf-2 inducers exerted an opposite effect on catalase, despite not significantly different from H_2O_2. On the other point of view, the use of reference compounds in our experimental settings was useful to remark that corn extracts could act through Nrf-2 independent mechanisms.

3.2.3. Effect of pigmented corn on epithelial integrity

The typical inflammatory process of CeD is not only correlated with epithelial atrophy, but also with enhanced barrier permeability at gut level (Ramirez-Sanchez et al., 2020). The latter favors the translocation of gliadin-derived peptides across the lamina propria, thus amplifying the inflammatory response. Gliadin and pro-inflammatory cytokines targeting NF-κB are known to interfere with epithelial integrity in CaCo-2 cells, but the molecular mechanism was only partially clarified (Kaminsky et al., 2021; Van Buiten et al., 2018).

To address the potential role of corn extracts for epithelial integrity, we measured the variation of TEER in CaCo-2 cells grown in Transwell® plates. The pro-inflammatory cocktail used in previous experiments (IL-1β, IFN-γ, and digested gliadin) caused a relevant decrease of TEER value after 24 h, which was partially recovered by sodium butyrate (2 mM), used as reference compound (Huang et al., 2021). Corn extracts showed only a slight, not significant activity, which was preserved after

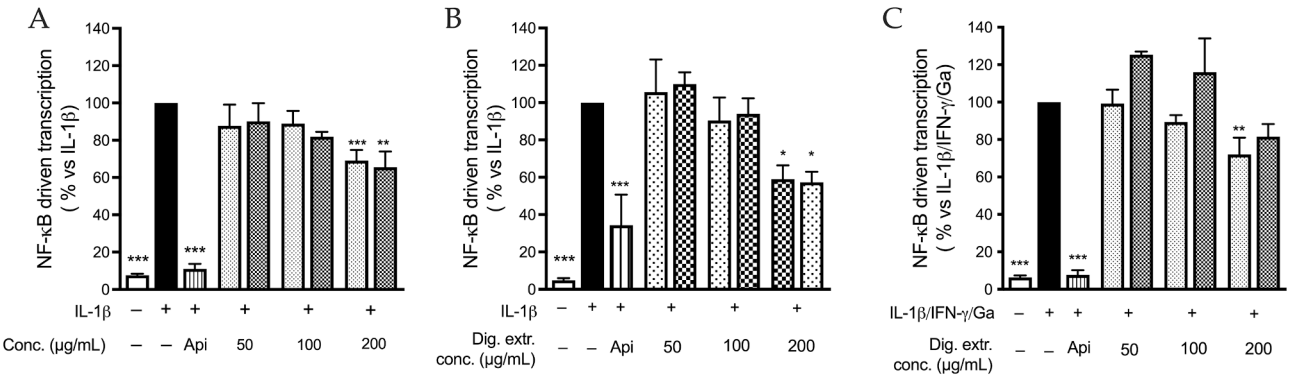

Fig. 4. Effect of pigmented corn extracts from varieties "Scagliolo Rosso" (dots) and "Rostrato di Rovetta" (squares) on the NF-κB driven transcription, measured by reporter plasmid. Caco-2 cells were treated for 6 h with IL-1β or pro-inflammatory cocktail (black bar) and extracts, which evaluated before (**A**) and after simulated digestion (**B**, **C**). Apigenin 20 μM (vertical lines) was used as reference inhibitor. Data were expressed as mean (% of luminescence units) ± SEM relative to IL-1β or pro-inflammatory cocktail (stimulus), to which was arbitrary assigned the value of 100 %. Dig. extr., digested extracts; Ga, digested gliadin; Api, apigenin; *p < 0.50, **p < 0.01, ***p < 0.001, p values vs stimulus.

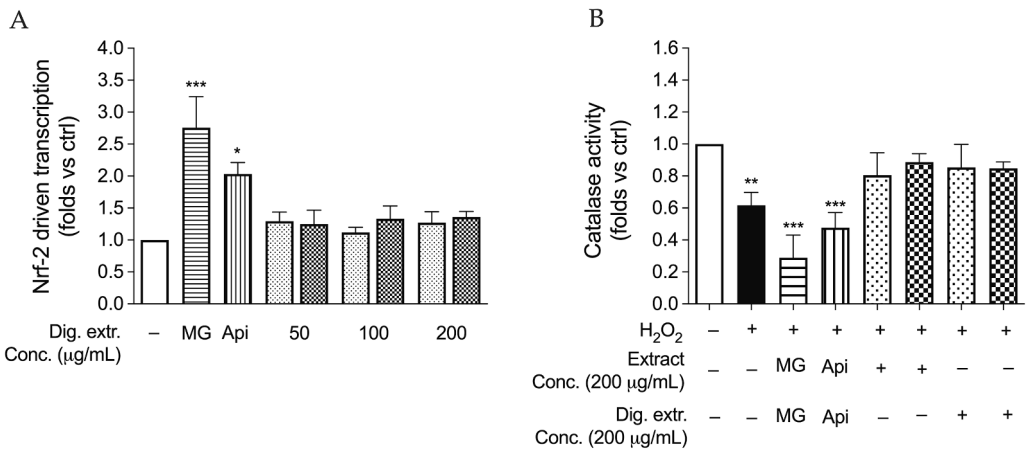

Fig. 6. Effect of pigmented corn extracts from varieties "Scagliolo Rosso" (dots) and "Rostrato di Rovetta" (squares) on the Nrf-2 driven transcription (**A**) and catalase activity (**B**). Caco-2 cells were treated for 24 h with extracts (**A**) followed by 1 h with H_2O_2 (black bar) (**B**). Apigenin 20 μM (vertical lines) and MG-132 1 μM (horizontal lines) were used as reference inducers of Nrf-2 driven transcription. Data were expressed as folds ± SEM relative to unstimulated control (white bar), to which was arbitrary assigned the value of 1. Dig. extr., digested extracts; Api, apigenin; MG, MG-132; *p < 0.50, **p < 0.01, ***p < 0.001, p values vs stimulus.

simulated digestion only for Scagliolo Rosso (Fig. 7).

4. Discussion

Cereals devoid of gluten, such as corn (*Zea mays* L.), are widely consumed by people with celiac disease (CeD). The pathogenesis of CeD is characterized by an autoimmune response, whose residual oxidative and inflammatory markers might persist even in subjects adopting gluten-free dietary protocols (Manavalan et al., 2010; Moretti et al., 2018; Odetti et al., 1998).

Our in vitro experiments investigated for the first time the role of Italian pigmented corns, belonging to the varieties Scagliolo Rosso and Rostrato di Rovetta, in intestinal inflammation and oxidative stress. The impact of the intestinal environment was considered; moreover, the polyphenolic composition was investigated to build a parallel with the biological activity.

Our analysis showed that Scagliolo Rosso and Rostrato di Rovetta retained relevant levels of phenols after simulated digestion (Tab. 1, TPC). Although not significant, these levels tended to increase for Scagliolo Rosso. Similarly, in a study performed by Ti and collaborators (2015) (Ti et al., 2015), in vitro simulated digestion resulted in an increase in the content of soluble polyphenols, probably related to the release of insoluble polyphenols and/or the action of digestive enzymes on phenols and proteins. Accordingly, the same authors reported an increase in antioxidant activity in brown rice samples of 110 % following digestion.

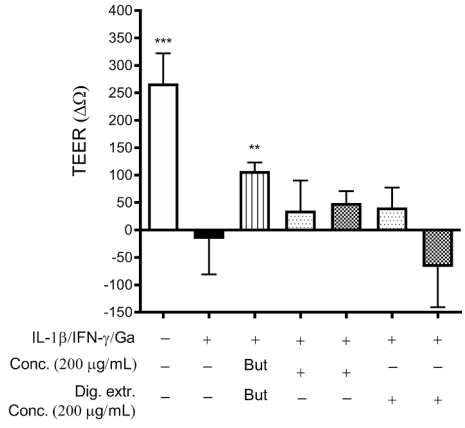

Fig. 7. Effect of pigmented corn extracts from varieties "Scagliolo Rosso" (dots) and "Rostrato di Rovetta" (squares) on epithelial integrity measured by TEER (Ω). Caco-2 cells were treated for 24 h (t_{24}) with corn extracts and pro-inflammatory cocktail (black bar, stimulus). Sodium butyrate 2 mM (vertical lines) was used as reference compound. Data were expressed as ΔΩ (Ωt_{24h}-Ωt_0) ± SEM. Dig. extr., digested extracts; Ga, digested gliadin; But, sodium butyrate 2 mM; **p < 0.01, ***p < 0.001, p values vs stimulus.

The determination of the scavenging capacity of the sample is usually influenced by the nature of the radical used in the colorimetric test (Zilic et al., 2012). Generally, an individual test is not enough to evaluate the reducing activity of all the compounds present in complex mixture because the sensitivity of radicals to the structures of phenolic compounds can vary. To obtain a more realistic evaluation, it is important to use different analytical methods (Prior et al., 2005). For this reason, the scavenging capacity of extracts was evaluated in parallel using two different tests: DPPH (1,1-diphenyl-2-picrylhydrazyl) and TEAC (Trolox Equivalent Antioxidant Capacity) assays.

The digestive process did not influence the scavenging capacity; in fact, the activity against DPPH and AAPH increased for both extracts (Tab. 1, AOA). In line with the phenolic content, the scavenging capacity was higher in Scagliolo Rosso than in Rostrato di Rovetta corn samples. Moreover, the HPTLC analysis (Fig. 1) showed that the phenolic profile of all samples changed after in vitro digestion. Accordingly, it was also characterized by a high antioxidant capacity after digestion, thus remarking the qualitative difference among corn samples and within samples after digestion.

Regarding the nature of phenols, Scagliolo Rosso extract was rich in anthocyanins, while this class of molecules was not detected in Rostrato di Rovetta, thus suggesting that other compounds, such as phlobaphenes, might account for the kernel color (Chatham et al., 2019). The total amount of anthocyanins was quantified, thus showing data comparable to Lopez-Martinez and collaborators (2009) (Lopez-Martinez et al., 2009), who found a total anthocyanin content between 0.015 and 8.50 mg CY/g in corns with different pigmentation. In several studies, the main anthocyanins identified in pigmented corn, along with their respective malonyl derivatives (de Pascual-Teresa et al., 2002; Yang & Zhai, 2010), are cyanidin, pelargonidin, and peonidin glycosides (Abdel-Aal el et al., 2006; Cuevas Montilla et al., 2011; Zilic et al., 2012). Our analysis is consistent with the findings reported in the literature, which identifies the primary compounds in pigmented corn as follows: cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-(6-malonylglucoside), peonidin-3-(6-malonylglucoside), and pelargonidin-3-(6-malonylglucoside). In fact, HPLC-DAD analysis (Fig. 2, Tab. 2) showed that cyanidin-3-O-glucoside is the most representative anthocyanin in Scagliolo Rosso, followed by cyanidin-3,6-malonylglucoside and peonidin-3-O-glucoside. The total

anthocyanin content ranged between 4.87 ± 0.07 and 3.73 ± 0.2 mg/g extract for Scagliolo Rosso before and after digestion, respectively. According to the spectrophotometric results, the anthocyanins showed a slight and significant decreased during in vitro digestion, and an overall reduction of 23.45 % was observed.

Of note, in the gastric environment, anthocyanins are known to remain stable (McDougall et al., 2005; Perez-Vicente et al., 2002). The stability of anthocyanins can be influenced by various factors, including pH, the presence of oxygen, their chemical structure, and the action of enzymes (Yousuf et al., 2016). Several studies in the literature have demonstrated that at the stomach's acidic pH (1–2), anthocyanins are found in their stable cationic form (McDougall et al., 2005; Perez-Vicente et al., 2002; Sangiovanni et al., 2015). Conversely, in the neutral pH environment of the intestine, due to the presence of the microbiota, anthocyanins undergo to conversion into various metabolites, such as phenolic acids and aldehyde compounds (Aura et al., 2005; McGhie & Walton, 2007). Obviously, the role of the microbiota represents a relevant limit of our study, in which microbial fermentation was not performed.

Nevertheless, the biological experiments conducted in CaCo-2 suggest that polyphenolic metabolites formed after simulated digestion might interfere with inflammatory signals, preferentially involved in the production of CXCL-10 and sICAM-1 (Fig. 3). Namely, these mediators were induced by a pro-inflammatory cocktail containing gliadin and are relevant for lymphocyte and macrophage recruitment in CeD (Ramirez-Sanchez et al., 2020). In particular, the activity of NF-κB was moderately impaired by corn extracts at the concentration of 200 μg/mL (Fig. 4B). However, Rostrato di Rovetta showed lower IC_{50} on CXCL-10 and sICAM-1 than Scagliolo Rosso, thus reminding to further targets involved in this effect.

Our results comply with other previous works which ascribe anti-inflammatory properties at gut level to phenolic acids and anthocyanins, known to impair the NF-κB pathway (Ferrari et al., 2016; Taverniti et al., 2014; Zhu et al., 2019). In addition, we excluded the involvement of a less investigated pathway like STAT-1; of note, other authors already excluded that pelargonidin (100 μM) could affect this pathway (Hamalainen et al., 2007).

The anti-inflammatory properties of corn extracts were not reflected in significant improvement of barrier integrity (Fig. 7). Of note, the same behavior was observed for the reference anti-inflammatory compound apigenin 20 μM, which was unable to act also as reference barrier protector (data not shown). For this reason, the trophic SCFA sodium butyrate was selected as reference modulator, known to protect the gut barrier through different mechanisms (Huang et al., 2021).

Data concerning the antioxidant activity suggest that corn extracts prevent ROS formation through a shared mechanism (Fig. 5). In fact, none of the extracts activated the Nrf-2 pathway till the highest concentration tested (Fig. 6): we explained our observation by considering that many polyphenols, including anthocyanins, are reported to enhance the Nrf-2 pathway at concentrations higher than 20 μM (Ferrari et al., 2016). Accordingly, our reference flavonoid (apigenin 20 μM) showed just a moderate activity at comparable concentrations. In fact, in our experiments, the highest concentration tested (200 μg/mL), with the highest phenolic content (SR, 63.63 mg/g), exposed intestinal cells to 1.26 μg/mL of total phenols. Thus, at low concentrations, polyphenols from corn could have probably acted through chemical scavenging of peroxyl radicals, in line with data obtained in cell-free tests. In analogy with anti-inflammatory investigations, the antioxidant effect was only partially explained by the amount of individual polyphenols occurring in corn extracts. We are prone to suppose that the quality of metabolites and their interaction after intestinal digestion may explain the whole bioactivity observed in our experimental conditions.

5. Conclusion

The in vitro antioxidant and anti-inflammatory effects of pigmented corn extracts were observed at concentrations (50–200 μg/mL) compliant with a potential consumption of corn-based foods. Further studies are required to understand the application of our evidence to human nutrition, such as the development of functional foods, or cooked and baked products, with possible impact from food technologies. Despite our data need to be validated by human studies, we suggest that pigmented corns might be valuable in gluten-free diet for the control of residual inflammatory and oxidative state in celiac disease.

CRediT authorship contribution statement

Stefano Piazza: Writing – original draft, Investigation, Data curation, Conceptualization. **Corinne Bani:** Investigation. **Francesca Colombo:** Investigation. **Francesca Mercogliano:** Investigation. **Carola Pozzoli:** Investigation. **Giulia Martinelli:** Investigation. **Katia Petroni:** Investigation. **Salvatore Roberto Pilu:** Investigation. **Elisa Sonzogni:** Investigation. **Marco Fumagalli:** Methodology, Investigation, Data curation. **Enrico Sangiovanni:** Writing – review & editing. **Patrizia Restani:** Writing – review & editing. **Mario Dell'Agli:** Writing – review & editing, Supervision, Conceptualization. **Chiara Di Lorenzo:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114640.

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