Health benefits of ancient grains. Comparison among bread made with ancient, heritage and modern grain flours in human cultured cells

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Abbreviations: AGE: advanced glycation end products; Ar: arginase; βCE: β-carotene equivalents; BSA: bovine serum albumin; CH: Choteau; cNOS: constitutive nitric oxide synthase; Ctrl: control; DCFH-DA: dichloro-dihydro-fluorescein diacetate; DMEM: Dulbecco's modified Eagle's medium; DPBS: Dulbecco's phosphate-buffered saline; ECACC: European Collection of Authenticated Cell Cultures; EDTA: ethylenediaminetetraacetic acid; eNOS: endothelial nitric oxide synthase; FBS: fetal bovine serum; FO: Fortuna-USA; GAE: gallic acid equivalent; HSD: honestly significant difference; IL-1β: interleukin-1β; IL-8: interleukin-8; IL-10: interleukin-10; iNOS: inducible nitric oxide synthase; JU: Judy; KA: KAMUT® khorasan wheat; LPS: lipopolysaccharides; MA: Marquis; MRP: Maillard reaction products; MTT: 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaS: sodium salicylate; NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; NOS: nitric oxide synthase; NSAID: nonsteroidal anti-inflammatory drugs; RE: Redwin; RNS: reactive nitrogen species; ROS: reactive oxygen species; SP: Spelt; TAC: total antioxidant capacity; TCC: total carotenoid content; TE: Trolox equivalents; TNF-α: tumor necrosis factor α; TPC: total phenolic content; TU: Turkey Red.

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ABSTRACT

Nowadays the higher nutritional value of whole grains compared to refined grains is recognized. In the last decade, there has been a renewed interest in the ancient wheat varieties for producing high-value food products with enhanced health benefits. This study compared two ancient grains, two heritage grains, and four modern grains grown in the same agronomic conditions considering not only their chemical characteristics, but also their biological effects. Whole grain flours were obtained and used to make bread. Bread was *in vitro* digested, the digesta were supplemented to HepG2 cells, and the biological effects of supplementation were evaluated. In addition, cells previously supplemented with the different digested bread types were then exposed to inflammatory agents to evidence possible protective effects of the pre-treatments. Despite the impossibility to discriminate bread made with different grains based on their chemical composition, results herein reported evidence that their supplementation to cultured cells exerts different effects, confirming the potential health benefits of ancient grains. This research represents an advancement for the evaluation of the apparent positive effects of ancient grains and the formulation of cereal-based products with added nutritional value.

Key words: ancient wheat; heritage wheat; modern wheat; KAMUT® khorasan wheat; spelt; cultured cells; antioxidants; inflammation.

1. Introduction

Food products derived from cereal grains constitute a major part of the daily diet, and wheat is the most important crop for humans (Shewry, 2009). Today most of the wheat species grown are hybrids which have been created from ancient wheat over the last 100 to 150 years. Although these "modern" wheat varieties have positive properties in terms of yield compared with the original ancient wheat, little attention has been given to their nutritional value because wheat quality has traditionally been judged on the basis of its technological functionality (Adom, Sorrells, & Liu, 2003; Serpen, Gökmen, Karagöz, & Köksel, 2008). In the last decade, there has been a renewed interest in the ancient varieties for producing high-value food products with enhanced health benefits (Bordoni, Danesi, Di Nunzio, Taccari, & Valli, 2017; Dinu, Whittaker, Pagliai, Benedettelli, & Sofi, 2018). These beneficial properties are ascribed to higher levels of proteins, lipids (mostly unsaturated fatty acids), soluble fibers, minerals, vitamins and phytochemicals (Dinelli et al., 2007; Hidalgo & Brandolini, 2014; Piergiovanni, Rizzi, Pannacciulli, & Gatta, 1997; Vrček et al., 2014). They are chiefly concentrated in the outer layers of grains (Adom, Sorrells, & Liu, 2005; Landberg, Kamal-Eldin, Salmenkallio-Marttila, Rouau, & Åman, 2008), which could explain the reduction of the risk of developing many diseases that is associated with higher whole grain consumption (Poutanen et al., 2008; Thorup, Gregersen, & Jeppesen, 2014). Nowadays, the higher nutritional value of whole grains than refined grains is recognized (Slavin, 2003), while the nutritional dominance of ancient *vs* modern grains is still controversial. In the literature, the most of the *in vitro* and animal studies

aimed to demonstrate the health benefit of ancient grains have been performed using extracts/lysates (Leoncini et al., 2012; Lucchesi et al., 2014; Whent et al., 2012) or discrete compounds derived from ancient wheat (Masisi et al., 2015). This represents a limitation since it is conceivable that the potential health benefit of ancient grains is not related to single compounds, but to their overall nutritional composition (Slavin, Jacobs, & Marquart, 2001). Furthermore, the use of extracts is far from reproducing the physiological situation, since grains undergo extensive treatment to produce foods, and foods must be digested before exerting any action into the body.

In addition, genetically-determined compositional differences existing among ancient and modern grains and varieties of the same species (Carvalho, Curto, & Guido, 2015; Righetti et al., 2016; Ziegler et al., 2015) are also affected by environmental factors (Danesi, Valli, Elementi, & D'Antuono, 2014; Menga, Fares, Troccoli, Cattivelli, & Baiano, 2010). Recently, Shewry (2017) highlighted that the most of studies comparing ancient and modern grains do not consider the interactions between genotype and environment, this hindering any comparison between ancient and modern grains.

In the attempt to further compare ancient and modern grain, we considered two ancient, two heritage, and four modern grains cultivated in the same location and growing season, using the same agronomic techniques. Whole grain flours were obtained from grains and bread was prepared using the same processing. The different bread types were characterized and compared, then they were *in vitro* digested and the ultra-filtered digesta were supplemented to HepG2 cells. To evidence whether the supplementation could exert a protective effect

toward a following inflammatory stimulus, in some experiments cells were pre-treated with the supplemented media and then submitted to an exogenous inflammatory stress. The effects of the supplementation were investigated by measuring cell viability, reactive oxygen species (ROS) and nitric oxide (NO) production, the expression of inducible nitric oxide synthase (iNOS) protein, and the secretion of a pro-inflammatory (IL-8) and an anti-inflammatory (IL-10) interleukin.

2. Material and Methods

2.1. Material

HepG2 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, UK). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were from Lonza (Basel, Switzerland). All other chemicals were from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals and solvents were of the highest analytical grade. Ingredients for bread formulation, except flour, were purchased at a local market.

2.2. Grains

Two ancient grains (KAMUT® khorasan wheat, KA; Spelt, SP), two heritage grains (Marquis, MA; Turkey Red, TU), and four modern grains (Choteau, CH; Fortuna-USA, FO; Judy, JU; Redwin, RE) were considered. Details about the different wheat varieties are from the Genetic Resources Information System for Wheat and Triticale website (GRIS, 2016). To minimize differences due to agronomic and environmental factors, all grains were organically cultivated in the same location and growing season. The eight wheat

varieties were planted after two years of green manure, one year of peas, one year of buckwheat. They were planted in May 2014 and harvested in August 2014.

Kernels were separated from the husk using a plot combine which was completely cleaned out between plots. The threshed grain was further cleaned from residues using sieves with different pores diameter. To obtain flour, cleaned grains were then milled with a small milling system (Molino Davide 4V, Novital; Lonate Pozzolo, Italy). After every grinding, each part of the milling system was carefully cleaned in order to avoid contamination, and flours were packed under vacuum and stored at 4°C.

2.3. Bread preparation

All bread types were made according to the same recipe (**Table 1**), limiting as much as possible the amount of other ingredients besides flour. A small-scale bread-maker (Pane Express, Ariete; Campi Bisenzio, Italy) was used to standardize the dough mixing and the baking steps; the same program (number 3) in the machine was set for all the preparations. Once baked, bread was cooled at room temperature, cut into pieces and stored at -20°C until analysis.

2.4. Bread nutritional composition and color analysis

Bread moisture, total nitrogen, carbohydrates, lipids, fibers, and ash were evaluated according to Baldini et al. (1996). Selenium concentration was determined by inductively coupled plasma-atomic emission spectrometry (Navarro-Blasco & Alvarez-Galindo, 2004). To evaluate the total antioxidant capacity (TAC) and the total phenolic content (TPC), 1 g of each bread was extracted according to Danesi et al. (2013) with a final volume of 6 mL

ethanol/water (70:30) acidified with 0.1% HCl. TAC was measured using the method of Re et al. (1999) and expressed as micromoles of Trolox equivalents (TE) per gram of bread. TPC was determined using Folin-Ciocalteu method, adapted to a 96-well plate assay according to Dicko et al. (2002). Results were expressed as mg gallic acid equivalent (GAE) per gram of bread.

The total carotenoid content (TCC) was determined using the method described by Valli et al. (2016) with some modifications. Briefly, 1 g of bread was mixed with 4 mL of hexane-acetone (50:50, v/v), shaken 20 min at 40°C, vortexed at high speed, sonicated, vortexed again, and centrifuged at 120 *g* for 3 min. The absorbance of the supernatants was measured at 450 nm and compared to the concentration–response curve of a β-carotene standard. Results were expressed as micrograms of β-carotene equivalents (βCE) per gram of bread.

The CIE system color profile of the eight bread samples was measured by a reflectance colorimeter (CR-400, Minolta; Milan, Italy) using illuminant source C (The International Commission on Illumination, 1978). Measurements were randomly taken at different locations in the bread samples. Results were expressed as values of the three-color components: L* the lightness (that ranges from 0 black to 100 white), a* the redness (that ranges from green associated with negative values to red associated with positive values) and b* the yellowness (that ranges from blue associated with negative values to yellow associated with positive values). The colorimeter was calibrated using a standard white ceramic tile.

2.5. *In vitro* digestion

149 Bread samples were digested according to the standardized method of Minekus et al.
150 (2014). The digestion process was performed on 50 g of experimental bread or 50 g of
151 water (blank digestion) for 240 min (120 min of gastric digestion and 120 min of intestinal
152 digestion) at 37°C. During the process, several consecutive enzymatic treatments took place
153 by addition of simulated saliva (containing 75 U/ml α-amylase), simulated gastric juice
154 (containing 2000 U/mL pepsin) at acid pH, and simulated pancreatic juice (containing 10
155 mM bile and 100 U/mL pancreatin) at neutral pH.
156 The digested solutions were centrifuged at 50,000 *g* for 15 min, and the supernatants
157 filtered with 0.2 µm membranes. To separate compounds which size is small enough to be
158 potentially absorbable through the intestinal mucosa, an aliquot was sequentially ultra-
159 filtered with Amicon Ultra at 3 kDa of molecular weight cut-off (Millipore; Billerica, MA,
160 USA) (<3kDa, bio-accessible fraction). Ultra-filtered solutions derived from two different
161 digestions of the same bread were mixed and frozen at -20°C until experiments. TAC and
162 TPC of the different bread digesta were determined as described above.

163 **2.6. HepG2 cells culture and supplementation**

164 HepG2 cells were maintained at 37°C, 95% air, 5% CO_2 in DMEM supplemented with
165 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin.
166 Once a week, cells were split 1:20 into a new 75 cm^2 flask, and the medium was refreshed
167 (Di Nunzio et al., 2017). Cells were seeded in 12-well plates at the concentration of 8×10^5
168 cells/mL. Cell counting was carried out using the TC20™ Automated Cell Counter (Bio-
169 Rad Laboratories; Hercules, CA, USA). After 24 h (75-80% confluence) cells were

incubated with DMEM without phenol red containing 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mg/mL BSA, 2 mM glutamine, and the <3kDa digested bread solutions at the concentration of 100 µL/mL. In preliminary experiments, scalar concentration of bread digesta were tested for cytotoxicity by the MTT assay (data not shown), and the 100 µL/mL concentration was the highest one having no toxic effect. Some cells were supplemented with 4 mM sodium salicylate (NaS) to compare the effect of digested bread to the effect of a well-known anti-inflammatory agent. NaS was supplemented to cell at a concentration that can be found in human plasma after therapeutic administration of the drug (Insel, 1996). To avoid interference due to the vehicle, some cells received a corresponding amount of a solution obtained from the "blank" digestion that is an *in vitro* digestion performed without food. Preliminary experiments were performed to check possible differences in terms of cell viability and cytokine secretion between cells receiving the "blank" digesta and cells receiving a corresponding amount of sterile water. No significant differences were observed (data not shown), so cells receiving the "blank" digesta were used as control (Ctrl).

After 24 hours (basal condition) media were removed, cells scraped-off and maintained at -20°C until analyses.

In some experiments, 24 h after supplementation media were removed and cells were incubated for two additional hours with new DMEM containing the inflammatory agent lipopolysaccharides (LPS, 100 ng/mL), interleukin-1β (IL-1β, 10 ng/mL), and tumor necrosis factor α (TNF-α, 10 ng/mL) (Hamidi et al., 2012). After additional 2 hours

191 (inflamed condition) media were removed, cells scraped-off and maintained at -20°C until
192 analyses. The experimental design is reported in **Figure 1**.

193 **2.7. Cell viability**

194 Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium
195 bromide (MTT) colorimetric assay, according to Di Nunzio et al. (2013). Briefly, cells were
196 washed twice with warm DPBS, then MTT dissolved in RPMI-1640 medium (final
197 concentration 0.5 mg/mL) was added to cells. After 1 h at 37°C, medium was completely
198 removed, 1-propanol was added to dissolve the formazan product, and the absorbance
199 measured against a propanol blank at 560 nm using a multiplate reader (Tecan Infinite
200 M200; Tecan, Männedorf, Switzerland). Results are expressed as percentage of the value
201 obtained in pair-matched Ctrl cells.

202 **2.8. ROS intracellular concentration**

203 Intracellular ROS concentration was monitored spectrofluorometrically as described in
204 detail by Valli et al. (2012). Briefly, dichloro-dihydro-fluorescein diacetate (DCFH-DA, 2
205 mM) in absolute ethanol was kept in the dark at -20°C until use. Ethanol final concentration
206 in the media was 1%, which has been already reported as not toxic (Wang et al., 2015). In
207 basal condition, 10 µL DCFH-DA/mL medium were added to HepG2 cells 30 min before
208 supplementation with digested bread. In inflamed condition, DCFH-DA at the same
209 concentration was added 30 min prior to the inflammatory stimulus. After 24 or 2 h
210 respectively, cells were washed twice with cold DPBS, lysed with 500 µL of cold Nonidet
211 P-40 (0.25% in DPBS), incubated on ice with shaking for 30 min and centrifuged at 14,000

212 *g* for 15 min. DCF fluorescence intensity was detected in supernatants (λ_{ex}=485 nm,
213 λ_{em}=535 nm) using an Infinite F200 microplate reader (Tecan; Männedorf, Switzerland),
214 normalized for protein content in the sample, and expressed as percent of value in Ctrl cells.

215 **2.9. Nitric oxide (NO) production**

216 NO production was assessed measuring the final products of NO metabolism, nitrite and
217 nitrate, in the cell media. The Nitrite/Nitrate Fluorometric Assay Kit (Cayman Chemical;
218 Ann Arbor, Michigan, USA) was used following the manufacturer's instruction. No
219 determination is based on a two-step reaction. In the first one, nitrates in the sample are
220 converted to nitrites by nitrate reductase. Then, 2,3-diaminonaphthalene and NaOH are
221 added to allow conversion of nitrites into a fluorescent product, 1(H)-naphthotriazole,
222 which is detected fluorimetrically (λ_{ex} = 360 nm, λ_{em} = 430 nm) and is proportional to NO_2^-
223 concentration.
224 Results were normalized for protein content in the well, and are expressed as nanomoles
225 NO/mL medium/mg protein.

226 **2.10. Cytokines secretion in the cell media**

227 The level of the pro-inflammatory IL-8 and the anti-inflammatory IL-10 was estimated in
228 cell media in basal condition and after cell treatment with the inflammatory agents by
229 AlphaLISA assay kits (Perkin Elmer Inc.; Waltham, MA, USA) using 96-microwell plates
230 (96 1/2 AreaPlate from Perkin Elmer Inc.) and an EnSpire™ plate reader (Perkin Elmer
231 Inc.), and following the manufacturer's instructions (Bielefeld-Sevigny, 2009). In the
232 AlphaLISA assay, a biotinylated anti-analyte antibody binds to the streptavidin-coated

donor beads while another anti-analyte antibody is conjugated to AlphaLISA acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the acceptor beads, resulting in a sharp peak of light emission at 615 nm. Results were normalized for protein content in the well, and are expressed in pg/mg protein.

2.11. iNOS protein expression

Whole cell lysate from HepG2 cell was obtained using 0.25% Nonidet P40 plus protease inhibitor cocktail (1 mM AEBSF, 800 nM aprotinin, 50 µM bestatin, 15 µM E64, 20 µM leupeptin, 10 µM pepstatin A, and 5 mM EDTA) (Life Technologies Inc.; Camarillo, CA, USA). Proteins in cell lysate (40 µg) were analyzed on 10% Mini-PROTEAN TGX Stain-Fee™ Gels (Bio-Rad Laboratories; Hercules, CA, USA), which are able to produce, after UV-induction, a stable, quantitative, and western blotting compatible protein fluorescent signal due to the reaction of the trihalocompound incorporated into gel formulations with the tryptophan residues contained in proteins (Kazmin, Edwards, Turner, Larson, & Starkey, 2002).

After electrophoresis (200 mV for 30 min), gel proteins were activated by UV exposure for 5 min, transferred onto a nitrocellulose membrane using a trans-blot turbo system (Bio-Rad Laboratories); protein fluorescence was acquired using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) with the Image Lab software (version 5.2.1). Proteins were then probed at room temperature for 60 min with the specific rabbit primary antibody anti-iNOS (1:1,000) (Life Technologies Inc.; Camarillo, CA, USA). After further washing, the

membrane was incubated with HRP-conjugated goat anti-rabbit IgG for 60 min (1:20,000) (Life Technologies Inc.; Camarillo, CA, USA). Final detection was performed with an enhanced chemiluminescence (ECL Prime) Western Blotting detection kit (GE Healthcare; Buckinghamshire, UK), and the images were acquired using the ChemiDoc™ MP Imaging System. Densitometry differences were analyzed with the Image Lab software and normalized for total fluorescent protein signal intensity.

2.12. Protein content

Cells were washed with cold DPBS, lysed with 500 µL of cold Nonidet P-40 (0.25% in DPBS), incubated on ice with shaking for 30 min and centrifuged at 14,000 *g* for 15 min. Supernatants were collected and stored at -20°C until protein determination. Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.13. Statistical analysis

All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's test or Tukey's honestly significant difference (HSD) test.

3. Results

3.1. Bread nutritional composition

The nutritional composition of the different flours is presented in **Table 2**. Bread made with MA, CH and FO grains had the highest content of total nitrogen, and bread made with MA and FO grains the lowest content of available carbohydrates. Water, lipids, ash, energy, and selenium content were similar among the different samples.

The bread color profile is reported in **Table 3**. The highest L* was detected in SP and JU samples, followed by TU. The ancient MA showed the highest a*, while KA the lowest. The highest b*value was detected in KA.

TAC, TPC, and TCC were species-specific, with no clear discrimination between ancient, heritage and modern grains (**Figure 2**). Overall, SP showed the highest TAC, TPC, and TCC. A significant positive correlation was observed between bread TAC and TPC (Pearson correlation coefficient: r^2=0.87; $p<0.001$), while no correlation was detected between bread TAC and TCC.

3.2. Digested bread

Digestion causes the release of compounds from the food matrix. Consequently, after *in vitro* digestion, both TAC and TPC were higher in the digesta than in the corresponding bread. Both parameters were similar in all digesta of all bread types, except in modern RE bread which showed significantly lower TAC (**Figure 3**). In the digesta, a significant positive correlation was observed between TAC and TPC (Pearson correlation coefficient r^2=0.57; $p<0.05$).

TCC in the digested fraction was below the detection limit, probably due to the low bioaccessibility of these molecules, as previously reported by other authors (Corte-Real, Richling, Hoffmann, & Bohn, 2014; Estévez-Santiago, Olmedilla-Alonso, & Fernández-Jalao, 2016).

3.3. Effects on cultured cells – basal condition

295 To evaluate the effect of bread digesta in basal condition, all markers were evaluated after
296 24 h supplementation. To avoid misleading results, cytotoxicity screening should be
297 considered mandatory before performing *in vitro* studies (Di Nunzio et al., 2017).
298 Accordingly, we used the MTT method to exclude any cytotoxic effect of the supplemented
299 digesta. Supplementation with bread digesta did not decrease cell viability, which appeared
300 higher in cells exposed to ancient grains than in controls. In contrast, NaS caused a
301 significant decrease in cell viability (**Figure 4A**).
302 Compared to controls, supplementation with all bread digesta except MA and FO, and with
303 NaS decreased ROS intracellular concentration (**Figure 4B**).
304 NO secretion in the cell media increased in cells supplemented with KA and TU (ancient
305 and heritage grain, respectively) and CH (modern grain), and mainly in NaS supplemented
306 cells (**Figure 4C**).
307 Compared to controls, secretion of pro-inflammatory IL-8 was significantly higher in 3 out
308 of 4 groups of cells supplemented with modern grain bread, and in cells supplemented with
309 NaS. On the contrary, IL-8 secretion was significantly lower in KA supplemented cells than
310 in controls (**Figure 5A**). In all cells, IL-10 secretion was very low, below the detection
311 limit.
312 No differences in iNOS protein expression were detected in supplemented cells compared
313 to control ones except in FO supplemented cells that evidenced a lower expression (**Figure**
314 **5B**).

315 **3.4. Effects on cultured cells – inflamed condition**

316 To evidence a possible protective effect exerted by the different bread digesta on a
317 following inflammatory stimulus, after 24 h supplementation media were changed, and
318 cells received fresh, not supplemented medium containing the inflammatory agents. All
319 markers were evaluated after 2 h inflammation.
320 In inflamed cells, no significant differences in cell viability were detected between control
321 and cells supplemented with bread digesta, and the detrimental effect of NaS was still
322 present (**Figure 6A**). Compared to control cells, ROS concentration was significantly
323 increased in all cells supplemented with bread made with modern grains, except CH ones
324 (**Figure 6B**). NO production was not influenced by the different supplementation except JU
325 and NaS, which caused a significant increase of NO concentration in the media (**Figure**
326 **6C**).
327 The pro-inflammatory stimulus greatly increased IL-8 production in all cells compared to
328 their basal counterparts. Compared to the control cells, IL-8 production was significantly
329 higher in NaS and modern grain supplemented cells except CH ones, while no differences
330 were detected among controls and cells supplemented with ancient and heritage grains
331 (**Figure 7A**). Even in inflamed condition, IL-10 secretion was below the detection limit.
332 No changes in iNOS protein expression were detected in supplemented cells compared to
333 controls (**Figure 7B**).

334 **4. Discussion**

335 To point out differences among the different grains, bread made with the corresponding
336 flours was characterized in term of nutritional composition, color profile, TAC, TPC, and

337 TCC. Analyses evidenced a substantial similarity of nutritional profiles and selenium
338 content among bread made with the different wheat varieties. Although these results are in
339 disagreement with a previous report (Gianotti et al., 2011), they are explained by the same
340 agronomic and environmental conditions in which grains were cultivated.
341 Differences among varieties were detected in the bread color profile. The color of plant
342 foods is mainly due to natural classes of pigment as carotenoids and anthocyanins. Several
343 studies have investigated the relationship between color and carotenoids (Fratianni, Irano,
344 Panfili, & Acquistucci, 2005; Hentschel et al., 2002) underlining that the degree of
345 yellowness in wheat grain and its end products is affected by carotenoids degradation
346 during processing (Ficco et al., 2014). Accordingly, in this study, the highest L* values
347 were detected in bread having the highest TCC, namely SP and JU bread.
348 According to Dinelli et al. (2011), a high variability of antiradical activity and phenolic
349 content were observed among the investigated bread, and a significant correlation was
350 detected between TAC and TPC, as reported by Adom & Lui (2002). Anyway, it was not
351 possible to discriminate ancient, heritage, and modern grains based on their TAC, TPC and
352 TCC values.
353 Digestion process and pH conditions result in starch hydrolysis, proteolysis and release of
354 phenolics from their conjugation forms as well as cell wall matrices (Li, Koecher, Hansen,
355 & Ferruzzi, 2016; Liyana-Pathirana & Shahidi, 2005; Szawara-Nowak, Bączek, &
356 Zieliński, 2016). Accordingly, bread *in vitro* digestion allowed the release of phenolic
357 substances from the food matrix, and an about 2-fold increase of TAC and TPC was

observed in the digesta compared to the corresponding undigested bread. Even in the digested fractions, a significant positive correlation was observed between TAC and TPC, but it was not possible a discrimination of different grains based on these parameters. The chemical characterization of foods is far from being a valid indicator of their nutritional and health value, and biological effects must be considered. Therefore, in the second part of the study we aimed to discriminate grains based on their effects when supplemented as digested bread to cultured liver cells. Hepatic cells were used as model system since they carry a nearly complete complement of xenobiotic metabolizing enzymes, and a subportion of the catalyzed reactions result in accumulation of metabolites that can cause either direct liver injury or indirect liver injury through activation of inflammation (Woolbright & Jaeschke, 2015). Furthermore, oxidative stress is considered as a conjoint pathological mechanism that contributes to initiation and progression of liver injury (Li et al., 2015), and the liver resembles a central organ of cytokine activity (Ramadori & Armbrust, 2001). HepG2 cells persist a large part of cellular functions like those of normal hepatocytes (Dehn, White, Conners, Shipkey, & Cumbo, 2004; Roe, Snawder, Benson, Roberts, & Casciano, 1993).

In basal condition, cell viability increased in cells supplemented with KAMUT® khorasan bread and spelt bread, while no effect of bread pre-supplementation was observed in inflamed cells. This allow excluding any effect on cell proliferation, since an increase in cell number during supplementation would affect viability also after inflammation. Since

the MTT assay evaluates the activity of mitochondrial dehydrogenase enzyme in living cells, our results seem mainly related to an increased cell vitality than cell number.

The about 20% decrease in cell viability observed in NaS supplemented cells compared to controls could be ascribed to the alteration in mitochondrial respiratory function already reported by Raza et al. (2011) in HepG2 cells. Since a similar decrease compared to pair-matched controls was observed also in inflamed condition, NaS effect could be also related to a cell cycle arrest and inhibition of cell proliferation (Raza et al., 2011).

The decrease in ROS production observed in almost all supplemented cells in basal condition is suggestive of a protective effect of the supplementation against oxidative stress. In inflamed condition, the protective effect observed in basal condition was not present anymore; on the contrary, the pre-treatment with the bread digesta significantly increased ROS concentration in cells supplemented with 3 out of 4 bread types made with modern grains (FO, RE, and JU), suggesting that the pre-exposure to modern grain digesta enhances the cell response to an inflammatory stimulus.

In basal condition, we observed an about 4-fold increase of NO concentration in KA, TU and CH supplemented cells, and a > 30-fold increase in cells exposed to NaS. The inflammatory stimulation increased NO production, that was similar in control and supplemented cells except JU and NaS ones.

Oxidative stress in the form of ROS or reactive nitrogen species (RNS) generation or disruption of the redox homeostasis in the cells is involved in cell signaling, self-defense, and apoptosis (Zhang et al., 2016). As a RNS, NO could initiate the oxidative stress.

Although RNS act together with ROS to damage cells, ROS generation and NO generation are not always induced in parallel. As example, silibinin has been reported to induce RNS generation without inducing ROS generation (Yu et al., 2012), and Huang et al. (2017) recently confirmed in pituitary GH3 cells that increased NO level can mediate the oxidative stress without any increase in the ROS level. Data herein reported further confirm the uncoupling of ROS and RSN generation.

NO is produced by nitric oxide synthase (NOS), which exists in three isoforms: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3). All of them share similar structures and catalytic modes, but they show different mechanisms regulating their expression and activities (Alderton, Cooper, & Knowles, 2001). The expression of NOS isoforms, including those constitutively expressed, may be triggered by different stimuli and in a tissue-dependent manner. Since in different cell types NaS (Callejas, Casado, Boscá, & Martín-Sanz, 2002) and phenolics (Costa, Francisco, Lopes, Cruz, & Batista, 2012; Vodovotz et al., 2004) have been reported to modulate NOS-2, the expression of NOS-2 protein was evaluated in the different experimental conditions. Unexpectedly, no modifications of NOS-2 protein expression were observed in supplemented cells compared to control ones, neither in basal nor in inflamed condition. The uncoupling of the observed increased in NO production to NOS-2 protein expression could be ascribed to the increased activity of another NOS isoform, NOS-3. In the liver, NO can be synthesized by the activity of any of the NOS isoforms, but the endothelial nitric oxide synthase (NOS-3) is the main source of endogenous NO (Rockey, 1997). NOS-3

activity can be regulated (Gonzalez-Rubio et al., 2016), and the use of molecular treatments associated with the increase of NOS-3 expression and activity has shown a beneficial effect for the liver (Biecker et al., 2008). Alternatively, the increased NO availability could be due to a decreased arginase (Ar) activity. Ar competes with NOS for the common substrate, L-arginine. Under normal conditions, NOS metabolizes L-arginine into L-citrulline, producing NO while Ar metabolizes L-arginine into L-ornithine and urea (Yang & Ming, 2014). Flavonoids have been reported to inhibit Ar activity in cultured endothelial cells, thus increasing NO production (Schnorr et al., 2008). Further studies are needed to unravel the mechanism at the basis of the observed increase in NO concentration.

Cytokines are the major local mediators of intercellular communications required to integrate the stimuli response in immune and inflammatory processes. IL-8 is a pro-inflammatory molecule inducing cytotoxic effects (Makni et al., 2011), whereas IL-10 is a prototypical regulatory cytokine exerting several immune-modulatory effects, and cereals have been shown to stimulate its production in monocytes (Yamazaki, Murray, & Kita, 2008). Since HepG2 cells have been reported to produce IL-8 and IL-10 in response to specific stimulation (Valli et al., 2016), these two cytokines were chosen as markers to further evaluate the possible modulation of inflammation by the different bread digesta. Interestingly, in basal condition supplementation with bread made with modern grains except RE increased IL-8 secretion. On the contrary, supplementation with KA reduced IL-8 level. Gliadin, a class of proteins that together with glutenins is the main component of the gluten fraction of the wheat seed, broadly induces cytokine (including IL-8) production

441 in cultured Caco-2 cells (Capozzi et al., 2013) and in peripheral blood mononuclear cells
442 from both patients with celiac disease and healthy controls (Lammers et al., 2011). The
443 concentration of gliadin proteins carrying allergenic epitopes among the total protein
444 pattern can influence the inflammatory response, and KAMUT® khorasan wheat has been
445 showed to have a lower percentage of epitopes than heritage and modern wheat (Valerii et
446 al., 2015). Results herein reported confirm a lower inflammatory potential of KAMUT®
447 khorasan wheat (Carnevali et al., 2014) than other tested grains. A higher concentration of
448 epitopes in gliadin protein of modern grains could also explain the observed additive effect
449 of the inflammatory stimulus and modern bread pre-supplementation on IL-8 secretion.
450 NaS supplementation (basal condition) or pre-supplementation (inflamed condition)
451 increased IL-8 secretion. A similar effect of salicylate in not-inflammatory condition has
452 been reported already in human skin fibroblasts (Ulrich-Merzenich et al., 2017). This could
453 be ascribed to the inability of nonsteroidal anti-inflammatory drugs, including NaS, to
454 interfere with NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells (Callejas et
455 al., 2002), which regulates the production of many pro-inflammatory cytokines, including
456 IL-8 (Roebuck, 1999).

457 **5. Conclusions**

458 Despite the impossibility to discriminate bread made with ancient, heritage or modern
459 grains based on their chemical composition, the effects exerted by their supplementation to
460 cultured cells were different. Different markers were used to evaluate the protective role of
461 bread and, in basal condition, ancient grains ameliorated the most of them. In inflamed

462 condition, the pre-treatment with most of the bread made with modern grains enhanced
463 ROS concentration and IL-8 production.
464 The positive activity of bread made with ancient wheat and the negative activity of bread
465 made with modern wheat could be due not only to compounds naturally present in grains
466 but also to increased browning reaction during baking and toasting processes (Slavin,
467 2003). It has been reported that some Maillard reaction products (MRP), in particular,
468 melanoidins, have beneficial effects as antioxidant (through the activation of the gene
469 expression of superoxide dismutase) and anti-inflammatory factors (de la Cueva, Seiquer,
470 Mesías, Rufián-Henares, & Delgado-Andrade, 2017; Delgado-Andrade, 2014). On the
471 other hand, other compounds generated during baking and toasting such as advanced
472 glycation end products (AGE) and acrylamide, are pro-inflammatory and toxic (Davis,
473 Prasad, Vijayagopal, Juma, & Imrhan, 2016; Zamani, Shaki, AbedianKenari, &
474 Shokrzadeh, 2017), and the final effect depends on the balance between positive and
475 negative molecules. Polyphenols can inhibit acrylamide formation (Liu et al., 2015), and it
476 is conceivable that the use of different flours led to a different production of MRP, AGE
477 and acrylamide, contributing to the different overall effect (Valli et al., 2016). Furthermore,
478 the structure of fiber matrix and the way the phenolic compounds inserted in the cereal
479 matrix strongly affect their physiological function. Studies *in vivo* (Mateo Anson,
480 Havenaar, Bast, & Haenen, 2010) and *in vitro* (Adam et al., 2002) after gastrointestinal
481 digestion displayed low bioavailability of ferulic acid in cereal cell walls, reflecting its
482 association with the fiber fraction through cross-linking with arabinoxylans and lignin.

Although this study does not allow to discriminate between protective and detrimental components, this must not be considered as a limitation since the possible synergism among the different molecules and the importance of some aspects related to the food matrix is known (Danesi, Govoni, D'Antuono, & Bordoni, 2016).

To the author's knowledge, the present study is the first one comparing ancient, heritage and modern grains grown in the same environment, so leaving out possible bias related to agronomic conditions. Overall, results herein reported confirm the potential health benefits of ancient grains. Although the use of *in vitro* digestion reduced in part the distance from the physiological situation *in vivo*, further investigations are needed to better understand how ancient grains contribute to the maintenance of human health. Until those studies are made, results herein reported highlight that ancient varieties could be useful in improving the nutritional value of cereal products, thereby stimulating producers to use these varieties in their current breeding strategies.

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REFERENCES

Adam, A., Crespy, V., Levrat-Verny, M. A., Leenhardt, F., Leuillet, M., Demigné, C., & Rémésy, C. (2002). The bioavailability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. *The Journal of Nutrition, 132*, 1962-1968.

Adom, K. K., & Liu, R. H. (2002). Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry, 50*, 6182-6187.

Adom, K. K., Sorrells, M. E., & Liu, R. H. (2003). Phytochemical profiles and antioxidant activity of wheat varieties. *Journal of Agricultural and Food Chemistry, 51*, 7825-7834.

Adom, K. K., Sorrells, M. E., & Liu, R. H. (2005). Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Chemistry, 53*, 2297-2306.

Alderton, W. K., Cooper, C. E., & Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *The Biochemical Journal, 357*, 593-615.

524 Baldini, M., Fabietti, F., Giammarioli, S., Onori, R., Orefice, L., & Stacchini, A. (1996).
525 *Analytical methods used in food chemical control [Metodi di analisi utilizzati per il*
526 *controllo chimico degli alimenti]*. Rome: Istituto Superiore di Sanità.
527 Biecker, E., Trebicka, J., Kang, A., Hennenberg, M., Sauerbruch, T., & Heller, J. (2008).
528 Treatment of bile duct-ligated rats with the nitric oxide synthase transcription enhancer
529 AVE 9488 ameliorates portal hypertension. *Liver International, 28*, 331-338.
530 Bielefeld-Sevigny, M. (2009). AlphaLISA immunoassay platform- the "no-wash" high-
531 throughput alternative to ELISA. *Assay and Drug Development Technologies, 7*, 90-92.
532 Bordoni, A., Danesi, F., Di Nunzio, M., Taccari, A., & Valli, V. (2017). Ancient wheat and
533 health: a legend or the reality? A review on KAMUT khorasan wheat. *International*
534 *Journal of Food Sciences and Nutrition, 68*, 278-286.
535 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram
536 quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,*
537 *72*, 248-254.
538 Callejas, N. A., Casado, M., Boscá, L., & Martín-Sanz, P. (2002). Absence of nuclear
539 factor κB inhibition by NSAIDs in hepatocytes. *Hepatology, 35*, 341-348.
540 Capozzi, A., Vincentini, O., Gizzi, P., Porzia, A., Longo, A., Felli, C., Mattei, V., Mainiero,
541 F., Silano, M., Sorice, M., & Misasi, R. (2013). Modulatory effect of gliadin peptide 10-
542 mer on epithelial intestinal Caco-2 cell inflammatory response. *PLoS One, 8*, e66561.
543 Carnevali, A., Gianotti, A., Benedetti, S., Tagliamonte, M. C., Primiterra, M., Laghi, L.,
544 Danesi, F., Valli, V., Ndaghijimana, M., Capozzi, F., Canestrari, F., & Bordoni, A. (2014).

545 Role of Kamut® brand khorasan wheat in the counteraction of non-celiac wheat sensitivity
546 and oxidative damage. *Food Research International, 63*, 218-226.
547 Carvalho, D. O., Curto, A. F., & Guido, L. F. (2015). Determination of phenolic content in
548 different barley varieties and corresponding malts by liquid chromatography-diode array
549 detection-electrospray ionization tandem mass spectrometry. *Antioxidants, 4*, 563-576.
550 Corte-Real, J., Richling, E., Hoffmann, L., & Bohn, T. (2014). Selective factors governing
551 *in vitro* β-carotene bioaccessibility: negative influence of low filtration cutoffs and
552 alterations by emulsifiers and food matrices. *Nutrition Research, 34*, 1101-1110.
553 Costa, G., Francisco, V., Lopes, M. C., Cruz, M. T., & Batista, M. T. (2012). Intracellular
554 signaling pathways modulated by phenolic compounds: application for new anti-
555 inflammatory drugs discovery. *Current Medicinal Chemistry, 19*, 2876-2900.
556 Danesi, F., Govoni, M., D'Antuono, L. F., & Bordoni, A. (2016). The molecular mechanism
557 of the cholesterol-lowering effect of dill and kale: The influence of the food matrix
558 components. *Electrophoresis, 37*, 1805-1813.
559 Danesi, F., Pasini, F., Caboni, M. F., D'Antuono, L. F., & Bordoni, A. (2013). Traditional
560 foods for health: screening of the antioxidant capacity and phenolic content of selected
561 Black Sea area local foods. *Journal of the Science of Food and Agriculture, 93*, 3595-3603.
562 Danesi, F., Valli, V., Elementi, S., & D'Antuono, L. F. (2014). The agronomic techniques
563 as determinants of the phenolic content and the biological antioxidant effect of palm-tree
564 kale. *Food and Nutrition Sciences, 5*, 1-7.

565 Davis, K. E., Prasad, C., Vijayagopal, P., Juma, S., & Imrhan, V. (2016). Advanced
566 glycation end products, inflammation, and chronic metabolic diseases: links in a chain?
567 *Critical Reviews in Food Science and Nutrition, 56*, 989-998.
568 de la Cueva, S. P., Seiquer, I., Mesías, M., Rufián-Henares, J. A., & Delgado-Andrade, C.
569 (2017). Evaluation of the availability and antioxidant capacity of Maillard compounds
570 present in bread crust: studies in Caco-2 cells. *Foods, 6*, article 5.
571 Dehn, P. F., White, C. M., Conners, D. E., Shipkey, G., & Cumbo, T. A. (2004).
572 Characterization of the human hepatocellular carcinoma (HepG2) cell line as an *in vitro*
573 model for cadmium toxicity studies. *In Vitro Cellular & Developmental Biology. Animal,*
574 *40*, 172-182.
575 Delgado-Andrade, C. (2014). Maillard reaction products: some considerations on their
576 health effects. *Clinical Chemistry and Laboratory Medicine, 52*, 53-60.
577 Di Nunzio, M., Toselli, M., Verardo, V., Caboni, M. F., & Bordoni, A. (2013).
578 Counteraction of oxidative damage by pomegranate juice: influence of the cultivar. *Journal*
579 *of the Science of Food and Agriculture, 93*, 3565-3573.
580 Di Nunzio, M., Valli, V., Tomás-Cobos, L., Tomás-Chisbert, T., Murgui-Bosch, L., Danesi,
581 F., & Bordoni, A. (2017). Is cytotoxicity a determinant of the different *in vitro* and *in vivo*
582 effects of bioactives? *BMC Complementary and Alternative Medicine, 17*, article 453.
583 Dicko, M. H., Hilhorst, R., Gruppen, H., Traore, A. S., Laane, C., van Berkel, W. J., &
584 Voragen, A. G. (2002). Comparison of content in phenolic compounds, polyphenol

oxidase, and peroxidase in grains of fifty sorghum varieties from Burkina Faso. *Journal of Agricultural and Food Chemistry, 50*, 3780-3788.

Dinelli, G., Marotti, I., Bosi, S., Benedettelli, S., Ghiselli, L., Cortacero-Ramírez, S., Carrasco-Pancorbo, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2007). Lignan profile in seeds of modern and old Italian soft wheat (*Triticum aestivum* L.) cultivars as revealed by CE-MS analyses. *Electrophoresis, 28*, 4212-4219.

Dinelli, G., Segura-Carretero, A., Di Silvestro, R., Marotti, I., Arráez-Román, D., Benedettelli, S., Ghiselli, L., & Fernández-Gutiérrez, A. (2011). Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography. A, 1218*, 7670-7681.

Dinu, M., Whittaker, A., Pagliai, G., Benedettelli, S., & Sofi, F. (2018). Ancient wheat species and human health: biochemical and clinical implications. *The Journal of Nutritional Biochemistry, 52*, 1-9.

Estévez-Santiago, R., Olmedilla-Alonso, B., & Fernández-Jalao, I. (2016). Bioaccessibility of provitamin A carotenoids from fruits: application of a standardised static *in vitro* digestion method. *Food & Function, 7*, 1354-1366.

Ficco, D. B. M., Mastrangelo, A. M., Trono, D., Borrelli, G. M., De Vita, P., Fares, C., Beleggia, R., Platani, C., & Papa, R. (2014). The colours of durum wheat: a review. *Crop and Pasture Science, 65*, 1-15.

Fratianni, A., Irano, M., Panfili, G., & Acquistucci, R. (2005). Estimation of color of durum wheat. Comparison of WSB, HPLC, and reflectance colorimeter measurements. *Journal of Agricultural and Food Chemistry, 53*, 2373-2378.

Gianotti, A., Danesi, F., Verardo, V., Serrazanetti, D. I., Valli, V., Russo, A., Riciputi, Y., Tossani, N., Caboni, M. F., Guerzoni, M. E., & Bordoni, A. (2011). Role of cereal type and processing in whole grain *in vivo* protection from oxidative stress. *Frontiers in Bioscience, 16*, 1609-1618.

Gonzalez-Rubio, S., Linares, C. I., Aguilar-Melero, P., Rodriguez-Peralvarez, M., Montero-Alvarez, J. L., de la Mata, M., & Ferrin, G. (2016). AAP-1 inhibition by SR 11302 protects human hepatoma HepG2 cells from bile acid-induced cytotoxicity by restoring the NOS-3 expression. *PLoS One, 11*, e0160525.

GRIS - Genetic Resources Information System for Wheat and Triticale (2016). Available online: http://wheatpedigree.net/ (accessed on 10 June 2016).

Hamidi, A., von Bulow, V., Hamidi, R., Winssinger, N., Barluenga, S., Heldin, C. H., & Landström, M. (2012). Polyubiquitination of transforming growth factor β (TGFβ)-associated kinase 1 mediates nuclear factor-κB activation in response to different inflammatory stimuli. *Journal of Biological Chemistry, 287*, 123-133.

Hentschel, V., Kranl, K., Hollmann, J., Lindhauer, M. G., Bohm, V., & Bitsch, R. (2002). Spectrophotometric determination of yellow pigment content and evaluation of carotenoids by high-performance liquid chromatography in durum wheat grain. *Journal of Agricultural and Food Chemistry, 50*, 6663-6668.

626 Hidalgo, A., & Brandolini, A. (2014). Nutritional properties of einkorn wheat (*Triticum*
627 *monococcum* L.). *Journal of the Science of Food and Agriculture, 94*, 601-612.
628 Huang, D., Cui, L., Guo, P., Xue, X., Wu, Q., Hussain, H. I., Wang, X., & Yuan, Z. (2017).
629 Nitric oxide mediates apoptosis and mitochondrial dysfunction and plays a role in growth
630 hormone deficiency by nivalenol in GH3 cells. *Scientific Reports, 7*, article 17079.
631 Insel, P. A. (1996). Analgesic-antipyretic and anti-inflammatory agents and drugs
632 employed in the treatment of gout. In J. G. Harman, P. B. Molinoff, R. W. Rudden, & A. G.
633 Gillman (Eds.), *The pharmacological basis of therapeutics* (pp. 617-657). New York, NY:
634 McGraw-Hill.
635 Kazmin, D., Edwards, R. A., Turner, R. J., Larson, E., & Starkey, J. (2002). Visualization
636 of proteins in acrylamide gels using ultraviolet illumination. *Analytical Biochemistry, 301*,
637 91-96.
638 Lammers, K. M., Khandelwal, S., Chaudhry, F., Kryszak, D., Puppa, E. L., Casolaro, V., &
639 Fasano, A. (2011). Identification of a novel immunomodulatory gliadin peptide that causes
640 interleukin-8 release in a chemokine receptor CXCR3-dependent manner only in patients
641 with coeliac disease. *Immunology, 132*, 432-440.
642 Landberg, R., Kamal-Eldin, A., Salmenkallio-Marttila, M., Rouau, X., & Åman, P. (2008).
643 Localization of alkylresorcinols in wheat, rye and barley kernels. *Journal of Cereal*
644 *Science, 48*, 401-406.

Leoncini, E., Prata, C., Malaguti, M., Marotti, I., Segura-Carretero, A., Catizone, P., Dinelli, G., & Hrelia, S. (2012). Phytochemical profile and nutraceutical value of old and modern common wheat cultivars. *PLoS One, 7*, e45997.

Li, M., Koecher, K., Hansen, L., & Ferruzzi, M. G. (2016). Phenolic recovery and bioaccessibility from milled and finished whole grain oat products. *Food & Function, 7*, 3370-3381.

Li, S., Tan, H. Y., Wang, N., Zhang, Z. J., Lao, L., Wong, C. W., & Feng, Y. (2015). The role of oxidative stress and antioxidants in liver diseases. *International Journal of Molecular Sciences, 16*, 26087-26124.

Liu, Y., Wang, P., Chen, F., Yuan, Y., Zhu, Y., Yan, H., & Hu, X. (2015). Role of plant polyphenols in acrylamide formation and elimination. *Food Chemistry, 186*, 46-53.

Liyana-Pathirana, C. M., & Shahidi, F. (2005). Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *Journal of Agricultural and Food Chemistry, 53*, 2433-2440.

Lucchesi, D., Russo, R., Gabriele, M., Longo, V., Del Prato, S., Penno, G., & Pucci, L. (2014). Grain and bean lysates improve function of endothelial progenitor cells from human peripheral blood: involvement of the endogenous antioxidant defenses. *PLoS One, 9*, e109298.

Makni, M., Chtourou, Y., Fetoui, H., Garoui el, M., Boudawara, T., & Zeghal, N. (2011). Evaluation of the antioxidant, anti-inflammatory and hepatoprotective properties of vanillin in carbon tetrachloride-treated rats. *European Journal of Pharmacology, 668*, 133-139.

Masisi, K., Diehl-Jones, W. L., Gordon, J., Chapman, D., Moghadasian, M. H., & Beta, T. (2015). Carotenoids of aleurone, germ, and endosperm fractions of barley, corn and wheat differentially inhibit oxidative stress. *Journal of Agricultural and Food Chemistry, 63*, 2715-2724.

Mateo Anson, N., Havenaar, R., Bast, A., & Haenen, G. R. M. M. (2010). Antioxidant and anti-inflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion. *Journal of Cereal Science, 51*, 110-114.

Menga, V., Fares, C., Troccoli, A., Cattivelli, L., & Baiano, A. (2010). Effects of genotype, location and baking on the phenolic content and some antioxidant properties of cereal species. *International Journal of Food Science & Technology, 45*, 7-16.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S., Weitschies, W., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food & Function, 5*, 1113-1124.

Navarro-Blasco, I., & Alvarez-Galindo, J. I. (2004). Selenium content of Spanish infant formulae and human milk: influence of protein matrix, interactions with other trace elements and estimation of dietary intake by infants. *Journal of Trace Elements in Medicine and Biology, 17*, 277-289.

687 Piergiovanni, A. R., Rizzi, R., Pannacciulli, E., & Gatta, C. D. (1997). Mineral composition
688 in hulled wheat grains: a comparison between emmer (*Triticum dicoccon* Schrank) and
689 spelt (*T. spelta* L.) accessions. *International Journal of Food Sciences and Nutrition, 48,*
690 381-386.
691 Poutanen, K., Shepherd, R., Shewry, P. R., Delcour, J. A., Björck, I., & Van Der Kamp, J.
692 W. (2008). Beyond whole grain: The European HEALTHGRAIN project aims at healthier
693 cereal foods. *Cereal Foods World, 53*, 32-35.
694 Ramadori, G., & Armbrust, T. (2001). Cytokines in the liver. *European Journal of*
695 *Gastroenterology & Hepatology, 13*, 777-784.
696 Raza, H., John, A., & Benedict, S. (2011). Acetylsalicylic acid-induced oxidative stress,
697 cell cycle arrest, apoptosis and mitochondrial dysfunction in human hepatoma HepG2 cells.
698 *European Journal of Pharmacology, 668*, 15-24.
699 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).
700 Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free*
701 *Radical Biology & Medicine, 26,* 1231-1237.
702 Righetti, L., Rubert, J., Galaverna, G., Folloni, S., Ranieri, R., Stranska-Zachariasova, M.,
703 Hajslova, J., & Dall'Asta, C. (2016). Characterization and discrimination of ancient grains:
704 A metabolomics approach. *International Journal of Molecular Sciences, 17*, article 1217.
705 Rockey, D. (1997). The cellular pathogenesis of portal hypertension: stellate cell
706 contractility, endothelin, and nitric oxide. *Hepatology, 25,* 2-5.

Roe, A. L., Snawder, J. E., Benson, R. W., Roberts, D. W., & Casciano, D. A. (1993). HepG2 cells: an in vitro model for P450-dependent metabolism of acetaminophen. *Biochemical and Biophysical Research Communications, 190*, 15-19.

Roebuck, K. A. (1999). Regulation of interleukin-8 gene expression. *Journal of Interferon & Cytokine Research, 19*, 429-438.

Schnorr, O., Brossette, T., Momma, T. Y., Kleinbongard, P., Keen, C. L., Schroeter, H., & Sies, H. (2008). Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo. *Archives of Biochemistry and Biophysics, 476*, 211-215.

Serpen, A., Gökmen, V., Karagöz, A., & Köksel, H. (2008). Phytochemical quantification and total antioxidant capacities of emmer (*Triticum dicoccon* Schrank) and einkorn (*Triticum monococcum* L.) wheat landraces. *Journal of Agricultural and Food Chemistry, 56*, 7285-7292.

Shewry, P. R. (2009). Wheat. *Journal of Experimental Botany, 60*, 1537-1553.

Shewry, P. R. (2017). Do ancient types of wheat have health benefits compared with modern bread wheat? *Journal of Cereal Science,* doi: 10.1016/j.jcs.2017.11.010

Slavin, J. (2003). Why whole grains are protective: biological mechanisms. *The Proceedings of the Nutrition Society, 62,* 129-134.

Slavin, J. L., Jacobs, D., & Marquart, L. (2001). Grain processing and nutrition. *Critical Reviews in Biotechnology, 21*, 49-66.

727 Szawara-Nowak, D., Bączek, N., & Zieliński, H. (2016). Antioxidant capacity and
728 bioaccessibility of buckwheat-enhanced wheat bread phenolics. *Journal of Food Science*
729 *and Technology, 53*, 621-630.
730 The International Commission on Illumination. (1978). *Recommendations on uniform color*
731 *spaces, color-difference equations, psychometric color terms*. Vienna, Austria: Commission
732 Internationale de l'Éclairage (CIE).
733 Thorup, A. C., Gregersen, S., & Jeppesen, P. B. (2014). Ancient wheat diet delays diabetes
734 development in a type 2 diabetes animal model. *The Review of Diabetic Studies, 11*, 245-
735 257.
736 Ulrich-Merzenich, G., Hartbrod, F., Kelber, O., Muller, J., Koptina, A., & Zeitler, H.
737 (2017). Salicylate-based phytopharmaceuticals induce adaptive cytokine and chemokine
738 network responses in human fibroblast cultures. *Phytomedicine, 34*, 202-211.
739 Valerii, M. C., Ricci, C., Spisni, E., Di Silvestro, R., De Fazio, L., Cavazza, E., Lanzini, A.,
740 Campieri, M., Dalpiaz, A., Pavan, B., Volta, U., & Dinelli, G. (2015). Responses of
741 peripheral blood mononucleated cells from non-celiac gluten sensitive patients to various
742 cereal sources. *Food Chemistry, 176*, 167-174.
743 Valli, V., Danesi, F., Gianotti, A., Di Nunzio, M., Taneyo Saa, D. L., & Bordoni, A.
744 (2016). Antioxidative and anti-inflammatory effect of *in vitro* digested cookies baked using
745 different types of flours and fermentation methods. *Food Research International, 88*, 256-
746 262.

747 Valli, V., Gómez-Caravaca, A. M., Di Nunzio, M., Danesi, F., Caboni, M. F., & Bordoni,
748 A. (2012). Sugar cane and sugar beet molasses, antioxidant-rich alternatives to refined
749 sugar. *Journal of Agricultural and Food Chemistry, 60*, 12508-12515.
750 Vodovotz, Y., Kim, P. K., Bagci, E. Z., Ermentrout, G. B., Chow, C. C., Bahar, I., &
751 Billiar, T. R. (2004). Inflammatory modulation of hepatocyte apoptosis by nitric oxide: *in*
752 *vivo*, *in vitro*, and in silico studies. *Current Molecular Medicine, 4*, 753-762.
753 Vrček, I. V., Čepo, D. V., Rašić, D., Peraica, M., Žuntar, I., Bojić, M., Mendaš, G., &
754 Medić-Šarić, M. (2014). A comparison of the nutritional value and food safety of
755 organically and conventionally produced wheat flours. *Food Chemistry, 143*, 522-529.
756 Wang, Y., Tong, J., Chang, B., Wang, B. F., Zhang, D., & Wang, B. Y. (2015). Effects of
757 ethanol on the expression of caveolin-1 in HepG2 cells. *Molecular Medicine Reports, 11*,
758 4409-4413.
759 Whent, M., Huang, H., Xie, Z., Lutterodt, H., Yu, L., Fuerst, E. P., Morris, C. F., Yu, L. L.,
760 & Luthria, D. (2012). Phytochemical composition, anti-inflammatory, and antiproliferative
761 activity of whole wheat flour. *Journal of Agricultural and Food Chemistry, 60*, 2129-2135.
762 Woolbright, B. L., & Jaeschke, H. (2015). Xenobiotic and endobiotic mediated interactions
763 between the cytochrome P450 system and the inflammatory response in the liver. *Advances*
764 *in Pharmacology, 74*, 131-161.
765 Yamazaki, K., Murray, J. A., & Kita, H. (2008). Innate immunomodulatory effects of cereal
766 grains through induction of IL-10. *The Journal of Allergy and Clinical Immunology, 121*,
767 172-178.e3.

768 Yang, Z., & Ming, X. F. (2014). Functions of arginase isoforms in macrophage
769 inflammatory responses: impact on cardiovascular diseases and metabolic disorders.
770 *Frontiers in Immunology, 5*, article 533.
771 Yu, Y., Fan, S. M., Yuan, S. J., Tashiro, S., Onodera, S., & Ikejima, T. (2012). Nitric oxide
772 (•NO) generation but not ROS plays a major role in silibinin-induced autophagic and
773 apoptotic death in human epidermoid carcinoma A431 cells. *Free Radical Research, 46*,
774 1346-1360.
775 Zamani, E., Shaki, F., AbedianKenari, S., & Shokrzadeh, M. (2017). Acrylamide induces
776 immunotoxicity through reactive oxygen species production and caspase-dependent
777 apoptosis in mice splenocytes via the mitochondria-dependent signaling pathways.
778 *Biomedicine & Pharmacotherapy, 94*, 523-530.
779 Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and
780 ROS-mediated cellular signaling. *Oxidative Medicine and Cellular Longevity, 2016*,
781 4350965.
782 Ziegler, J. U., Steingass, C. B., Longin, C. F. H., Würschum, T., Carle, R., & Schweiggert,
783 R. M. (2015). Alkylresorcinol composition allows the differentiation of *Triticum* spp.
784 having different degrees of ploidy. *Journal of Cereal Science, 65*, 244-251.
785

FIGURE LEGENDS

Figure 1. Scheme of experimental design and timeline in (A) basal and (B) inflamed condition.

Figure 2. (A) Total antioxidant capacity (TAC), (B) total phenolic content (TPC), and (C) total carotenoid content (TCC) of bread made with the different grains. Data are means ± SD (n=3). Panel A: Results are expressed as micromoles of Trolox equivalents (TE) per gram of bread. Panel B: Results are expressed as μmoles of mg gallic acid equivalent (GAE) per gram of bread. Panel C: Results are expressed as β-carotene equivalents (βCE) per gram of bread. Statistical analysis was by one-way ANOVA ($p<0.001$) followed by Tukey's test. Different letters indicate significant differences (at least $p<0.05$).

Figure 3. (A) Total antioxidant capacity (TAC) and (B) total phenolic content (TPC) of digested bread made with the different grains. Data are means ± SD (n=3). Panel A: Results are expressed as micromoles of Trolox equivalents (TE) per gram of bread. Panel B: Results are expressed as μmoles of mg gallic acid equivalent (GAE) per gram of bread. Statistical analysis was by one-way ANOVA ($p<0.001$) followed by Tukey's test. Different letters indicate significant differences (at least $p<0.05$).

Figure 4. (A) Cell viability, (B) ROS intracellular concentration, and (C) NO secretion in cells in basal condition. Results are means ± SD (n=6). Panel A: Results are expressed as percent of value in the control cells (assigned as 100%). Panel B: Results were normalized for protein content in the sample, and are expressed as percent of value in the control cells (assigned as 100%). Panel C: Results are expressed as nmol NO/mL medium/mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$) followed by Dunnett's test: * $p<0.05$ and *** $p<0.001$ *vs* control cells.

Figure 5. (A) Interleukin-8 (IL-8) secretion in the cell media and (B) iNOS protein relative expression in cell lysates in basal condition. Data are means ± SD (panel A: n=6; panel B: n=4). Panel A: Results are expressed as pg/mL medium/mg protein in the well. Panel B: Results were normalized for total fluorescent protein signal intensity, and are expressed as percent of value in the control cells (assigned as 100%). Statistical analysis was by one-way ANOVA (panel A: $p<0.001$; panel B: $p<0.01$) followed by Dunnett's test: * $p<0.05$ and *** $p<0.001$ *vs* control cells.

Figure 6. (A) Cell viability, (B) ROS intracellular concentration, and (C) NO secretion in cells in inflamed condition. Results are means ± SD (n=6). Panel A: Results are expressed as percent of value in the control cells (assigned as 100%). Panel B: Results were normalized for protein content in the sample, and are expressed as percent of value in the control cells (assigned as 100%). Panel C: Results are expressed as nmol NO/mL

828 medium/mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$)
829 followed by Dunnett's test: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ *vs* control cells.
830
831 **Figure 7. (A) Interleukin-8 (IL-8) secretion in the cell media and (B) iNOS protein**
832 **relative expression in cell lysates in inflamed conditions.** Data are means ± SD (panel A:
833 n=6; panel B: n=4). Panel A: Results are expressed as pg/mL medium/mg protein in the
834 well. Panel B: Results were normalized for total fluorescent protein signal intensity, and are
835 expressed as percent of value in the control cells (assigned as 100%). Statistical analysis
836 was by one-way ANOVA (panel A: $p<0.001$; panel B: n.s., not significant) followed by
837 Dunnett's test: * $p<0.05$, and *** $p<0.001$ *vs* control cells.