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PII: S0963-9969(18)30125-X
DOI: doi:10.1016/j.foodres.2018.02.032
Reference: FRIN 7395

To appear in: *Food Research International*

Received date: 20 October 2017
Revised date: 9 February 2018
Accepted date: 11 February 2018

Please cite this article as: Veronica Valli, Annalisa Taccari, Mattia Di Nunzio, Francesca Danesi, Alessandra Bordoni , Health benefits of ancient grains. Comparison among bread made with ancient, heritage and modern grain flours in human cultured cells. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Frin(2017), doi:10.1016/j.foodres.2018.02.032

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

**Veronica Valli [1,§], Annalisa Taccari [1], Mattia Di Nunzio [1], Francesca Danesi [1], Alessandra Bordoni [1,*]**

[1] Department of Agri-Food Sciences and Technologies (DISTAL), University of Bologna, Piazza Goidanich 60, 47521 Cesena, Italy.

[§] Present address: Sociedad Española de Colorantes Naturales y Afines (SECNA), Polígono 33, Parcela 254, El Muladar, 46370 Chiva, Valencia, Spain.

*[1] Corresponding author: Alessandra Bordoni

[1] **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.

Campus of Food Science, University of Bologna

Piazza Goidanich, 60 - 47521 Cesena (FC) Italy

Phone: +39 0547 338955

Fax: +39 0547 382348

e-mail: alessandra.bordoni@unibo.it

Authors' e-mail addresses:

| | |
|---|---|
| Veronica Valli | valli.veron@gmail.com |
| Annalisa Taccari | annalisa.taccari@gmail.com |
| Mattia Di Nunzio | mattia.dinunzio@unibo.it |
| Francesca Danesi | francesca.danesi@unibo.it |
| Alessandra Bordoni | alessandra.bordoni@unibo.it |

**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

Bread samples were digested according to the standardized method of Minekus et al. (2014). The digestion process was performed on 50 g of experimental bread or 50 g of water (blank digestion) for 240 min (120 min of gastric digestion and 120 min of intestinal digestion) at 37°C. During the process, several consecutive enzymatic treatments took place by addition of simulated saliva (containing 75 U/ml α-amylase), simulated gastric juice (containing 2000 U/mL pepsin) at acid pH, and simulated pancreatic juice (containing 10 mM bile and 100 U/mL pancreatin) at neutral pH.

The digested solutions were centrifuged at 50,000 *g* for 15 min, and the supernatants filtered with 0.2 µm membranes. To separate compounds which size is small enough to be potentially absorbable through the intestinal mucosa, an aliquot was sequentially ultra-filtered with Amicon Ultra at 3 kDa of molecular weight cut-off (Millipore; Billerica, MA, USA) (<3kDa, bio-accessible fraction). Ultra-filtered solutions derived from two different digestions of the same bread were mixed and frozen at -20°C until experiments. TAC and TPC of the different bread digesta were determined as described above.

**2.6. HepG2 cells culture and supplementation**

HepG2 cells were maintained at 37°C, 95% air, 5% $CO_2$ in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. Once a week, cells were split 1:20 into a new 75 $cm^2$ flask, and the medium was refreshed (Di Nunzio et al., 2017). Cells were seeded in 12-well plates at the concentration of $8 \times 10^5$ cells/mL. Cell counting was carried out using the TC20™ Automated Cell Counter (Bio-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

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

### 2.7. Cell viability

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

### 2.8. ROS intracellular concentration

Intracellular ROS concentration was monitored spectrofluorometrically as described in detail by Valli et al. (2012). Briefly, dichloro-dihydro-fluorescein diacetate (DCFH-DA, 2 mM) in absolute ethanol was kept in the dark at -20°C until use. Ethanol final concentration in the media was 1%, which has been already reported as not toxic (Wang et al., 2015). In basal condition, 10 µL DCFH-DA/mL medium were added to HepG2 cells 30 min before supplementation with digested bread. In inflamed condition, DCFH-DA at the same concentration was added 30 min prior to the inflammatory stimulus. After 24 or 2 h respectively, cells were washed twice 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. DCF fluorescence intensity was detected in supernatants ($\lambda_{ex}$=485 nm, $\lambda_{em}$=535 nm) using an Infinite F200 microplate reader (Tecan; Männedorf, Switzerland), normalized for protein content in the sample, and expressed as percent of value in Ctrl cells.

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

NO production was assessed measuring the final products of NO metabolism, nitrite and nitrate, in the cell media. The Nitrite/Nitrate Fluorometric Assay Kit (Cayman Chemical; Ann Arbor, Michigan, USA) was used following the manufacturer's instruction. No determination is based on a two-step reaction. In the first one, nitrates in the sample are converted to nitrites by nitrate reductase. Then, 2,3-diaminonaphthalene and NaOH are added to allow conversion of nitrites into a fluorescent product, 1(H)-naphthotriazole, which is detected fluorimetrically ($\lambda_{ex}$ = 360 nm, $\lambda_{em}$ = 430 nm) and is proportional to $NO_2^-$ concentration.

Results were normalized for protein content in the well, and are expressed as nanomoles NO/mL medium/mg protein.

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

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

To evaluate the effect of bread digesta in basal condition, all markers were evaluated after 24 h supplementation. To avoid misleading results, cytotoxicity screening should be considered mandatory before performing *in vitro* studies (Di Nunzio et al., 2017). Accordingly, we used the MTT method to exclude any cytotoxic effect of the supplemented digesta. Supplementation with bread digesta did not decrease cell viability, which appeared higher in cells exposed to ancient grains than in controls. In contrast, NaS caused a significant decrease in cell viability (**Figure 4A**).

Compared to controls, supplementation with all bread digesta except MA and FO, and with NaS decreased ROS intracellular concentration (**Figure 4B**).

NO secretion in the cell media increased in cells supplemented with KA and TU (ancient and heritage grain, respectively) and CH (modern grain), and mainly in NaS supplemented cells (**Figure 4C**).

Compared to controls, secretion of pro-inflammatory IL-8 was significantly higher in 3 out of 4 groups of cells supplemented with modern grain bread, and in cells supplemented with NaS. On the contrary, IL-8 secretion was significantly lower in KA supplemented cells than in controls (**Figure 5A**). In all cells, IL-10 secretion was very low, below the detection limit.

No differences in iNOS protein expression were detected in supplemented cells compared to control ones except in FO supplemented cells that evidenced a lower expression (**Figure 5B**).

### 3.4. Effects on cultured cells – inflamed condition

To evidence a possible protective effect exerted by the different bread digesta on a following inflammatory stimulus, after 24 h supplementation media were changed, and cells received fresh, not supplemented medium containing the inflammatory agents. All markers were evaluated after 2 h inflammation.

In inflamed cells, no significant differences in cell viability were detected between control and cells supplemented with bread digesta, and the detrimental effect of NaS was still present (**Figure 6A**). Compared to control cells, ROS concentration was significantly increased in all cells supplemented with bread made with modern grains, except CH ones (**Figure 6B**). NO production was not influenced by the different supplementation except JU and NaS, which caused a significant increase of NO concentration in the media (**Figure 6C**).

The pro-inflammatory stimulus greatly increased IL-8 production in all cells compared to their basal counterparts. Compared to the control cells, IL-8 production was significantly higher in NaS and modern grain supplemented cells except CH ones, while no differences were detected among controls and cells supplemented with ancient and heritage grains (**Figure 7A**). Even in inflamed condition, IL-10 secretion was below the detection limit. No changes in iNOS protein expression were detected in supplemented cells compared to controls (**Figure 7B**).

## 4. Discussion

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

TCC. Analyses evidenced a substantial similarity of nutritional profiles and selenium content among bread made with the different wheat varieties. Although these results are in disagreement with a previous report (Gianotti et al., 2011), they are explained by the same agronomic and environmental conditions in which grains were cultivated.

Differences among varieties were detected in the bread color profile. The color of plant foods is mainly due to natural classes of pigment as carotenoids and anthocyanins. Several studies have investigated the relationship between color and carotenoids (Fratianni, Irano, Panfili, & Acquistucci, 2005; Hentschel et al., 2002) underlining that the degree of yellowness in wheat grain and its end products is affected by carotenoids degradation during processing (Ficco et al., 2014). Accordingly, in this study, the highest L* values were detected in bread having the highest TCC, namely SP and JU bread.

According to Dinelli et al. (2011), a high variability of antiradical activity and phenolic content were observed among the investigated bread, and a significant correlation was detected between TAC and TPC, as reported by Adom & Lui (2002). Anyway, it was not possible to discriminate ancient, heritage, and modern grains based on their TAC, TPC and TCC values.

Digestion process and pH conditions result in starch hydrolysis, proteolysis and release of phenolics from their conjugation forms as well as cell wall matrices (Li, Koecher, Hansen, & Ferruzzi, 2016; Liyana-Pathirana & Shahidi, 2005; Szawara-Nowak, Bączek, & Zieliński, 2016). Accordingly, bread *in vitro* digestion allowed the release of phenolic 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

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

## 5. Conclusions

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

condition, the pre-treatment with most of the bread made with modern grains enhanced ROS concentration and IL-8 production.

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

**Funding:** This work was supported by Kamut Enterprises of Europe (Oudenaarde, Belgium). KAMUT® is a registered trademark of Kamut International, Ltd. and Kamut Enterprises of Europe, bvba.

**Author Contributions:** A.B. conceived and designed the experiments; V.V., A.T., M.D.N., and F.D. performed the experiments; all authors read and approved the final article.

**Conflicts of Interest:** All authors state that they have no conflicts of interest to declare that are related to this study. The founding sponsor has no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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## 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

medium/mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$) followed by Dunnett's test: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ *vs* control cells.

**Figure 7. (A) Interleukin-8 (IL-8) secretion in the cell media and (B) iNOS protein relative expression in cell lysates in inflamed conditions.** 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: n.s., not significant) followed by Dunnett's test: * $p<0.05$, and *** $p<0.001$ *vs* control cells.

## TABLES

**Table 1. Bread recipe.**

| Ingredient | Absolute quantity (g) | Relative quantity (%) |
|---|---|---|
| Flour | 400 | 57.5 |
| Water | 250 | 35.9 |
| Sugar | 15 | 2.2 |
| Salt | 3 | 0.4 |
| Dry yeast | 28 | 4.0 |

**Table 2. Nutritional composition and selenium content of the bread made with the different grains.** Data are means ± SD (n=3). Statistical analysis was carried out by the one-way ANOVA followed by Tukey's test (total nitrogen $p<0.001$, carbohydrates $p<0.01$, fibers $p<0.05$). Different letters in the same row indicate statistically significant differences (at least $p<0.05$).

| Component | Bread made with ancient wheat | | Bread made with heritage wheat | | Bread made with modern wheat | | | |
|---|---|---|---|---|---|---|---|---|
| | KA | SP | MA | TU | CH | FO | JU | RE |
| Water (g/100g) | 28.0 ± 1.4 $^{a}$ | 26.8 ± 1.3 $^{a}$ | 30.2 ± 1.5 $^{a}$ | 30.7 ± 1.5 $^{a}$ | 28.9 ± 1.5 $^{a}$ | 29.7 ± 1.5 $^{a}$ | 29.7 ± 1.5 $^{a}$ | 29.2 ± 1.5 $^{a}$ |
| Total Nitrogen (g/100g) | 12.7 ± 0.6 $^{c,d}$ | 13.4 ± 0.7 $^{b,c}$ | 15.4 ± 0.8 $^{a}$ | 12.6 ± 0.6 $^{c,d}$ | 14.9 ± 0.8 $^{a,b}$ | 15.3 ± 0.8 $^{a}$ | 12.2 ± 0.6 $^{d}$ | 11.3 ± 0.6 $^{d}$ |
| Carbohydrates (g/100g) | 49.9 ± 1.7 $^{a}$ | 49.7 ± 1.7 $^{a,b}$ | 42.7 ± 1.8 $^{c}$ | 47.1 ± 1.8 $^{a,b,c}$ | 45.2 ± 1.8 $^{a,b,c}$ | 44.8 ± 1.8 $^{b,c}$ | 47.9 ± 1.8 $^{a,b}$ | 49.1 ± 1.7 $^{a,b}$ |
| Lipids (g/100g) | 1.7 ± 0.2 $^{a}$ | 1.8 ± 0.2 $^{a}$ | 1.7 ± 0.2 $^{a}$ | 1.6 ± 0.2 $^{a}$ | 1.5 ± 0.2 $^{a}$ | 1.5 ± 0.2 $^{a}$ | 1.6 ± 0.2 $^{a}$ | 1.6 ± 0.2 $^{a}$ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Fibers (g/100g)** | 5.9 ± 0.6 [b] | 6.2 ± 0.6 [a,b] | 8.0 ± 0.8 [a] | 6.1 ± 0.6 [a,b] | 7.3 ± 0.7 [a,b] | 6.6 ± 0.7 [a,b] | 6.4 ± 0.6 [a,b] | 6.7 ± 0.7 [a,b] |
| **Ash (g/100g)** | 2.0 ± 0.3 [a] | 2.2 ± 0.3 [a] | 2.1 ± 0.3 [a] | 1.9 ± 0.3 [a] | 2.1 ± 0.3 [a] | 2.2 ± 0.3 [a] | 2.3 ± 0.3 [a] | 2.1 ± 0.3 [a] |
| **Energy (Kcal/100g)** | 277 [a] | 280 [a] | 263 [a] | 265 [a] | 269 [a] | 267 [a] | 268 [a] | 269 [a] |
| **Selenium (µg/100g)** | 56 ± 20 [a] | 79 ± 10 [a] | 34 ± 14 [a] | 45 ± 19 [a] | 34 ± 14 [a] | 40 ± 17 [a] | 52 ± 22 [a] | 54 ± 23 [a] |

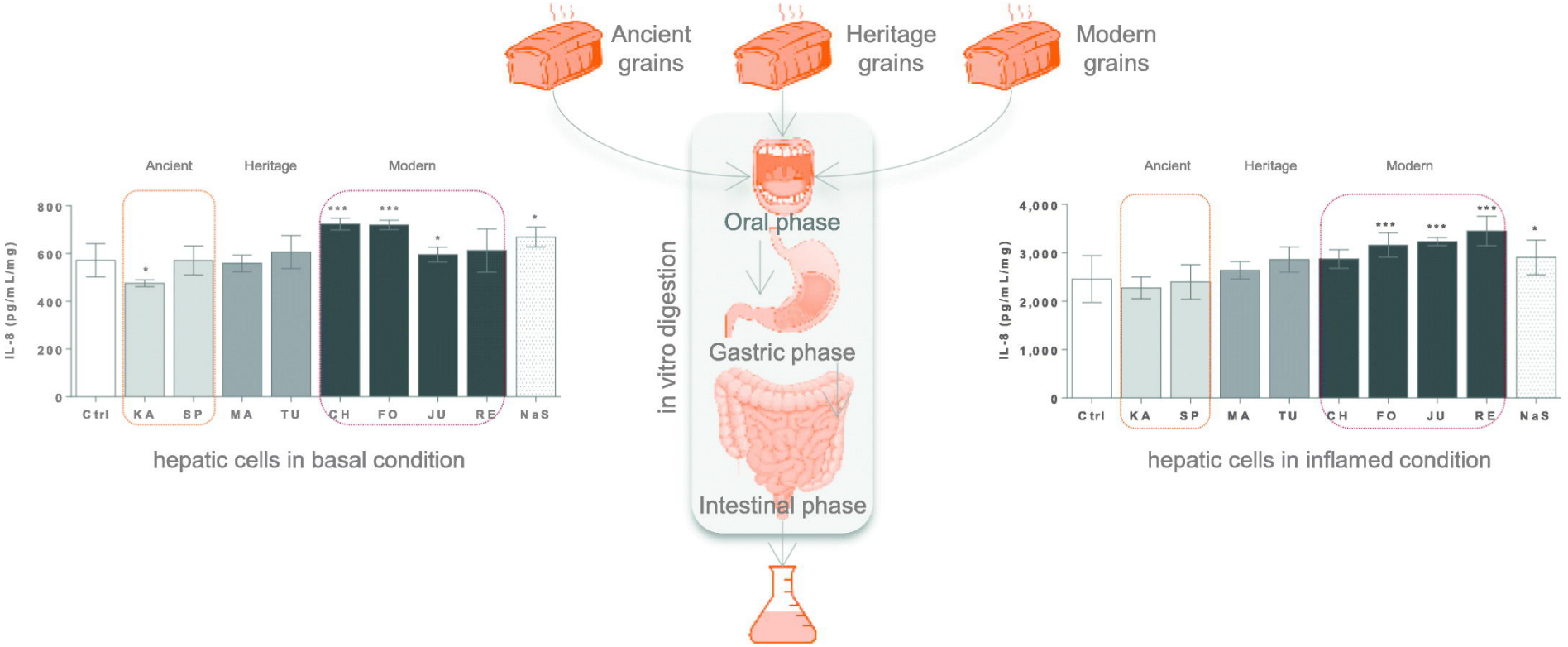
**Table 3. Bread color profile.** Data are means ± SD (n=3). Statistical analysis was by one-way ANOVA ($p<0.001$) followed by Tukey's test.

Different letters indicate significant differences (at least $p<0.05$).

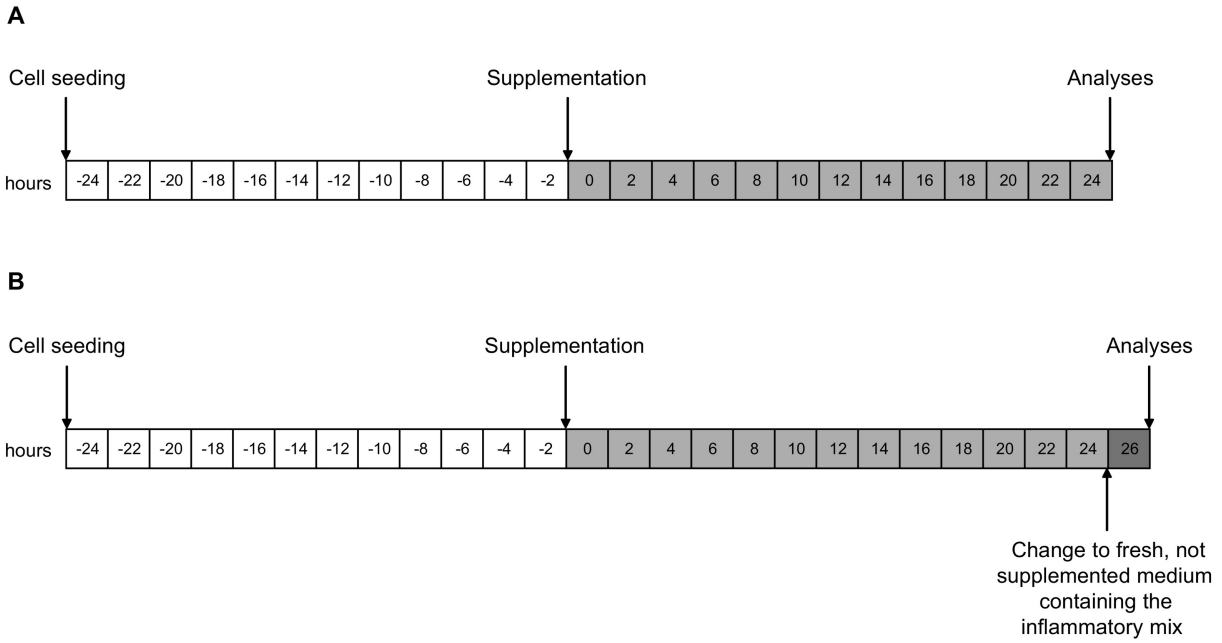
| Color component | Bread made with ancient wheat | | Bread made with heritage wheat | | Bread made with modern wheat | | | |
|---|---|---|---|---|---|---|---|---|
| | KA | SP | MA | TU | CH | FO | JU | RE |
| **L*** | 49.8 ± 1.2 $^{d,e}$ | 57.6 ± 0.8 $^{a}$ | 47.6 ± 0.3 $^{e,f}$ | 55.4 ± 1.3 $^{a,b}$ | 46.5 ± 1.2 $^{f}$ | 52.2 ± 2.7 $^{c,d}$ | 57.3 ± 0.0 $^{a}$ | 54.0 ± 0.0 $^{b,c}$ |
| **a*** | 3.5 ± 0.2 $^{f}$ | 4.8 ± 0.3 $^{d,e}$ | 6.2 ± 0.0 $^{a}$ | 5.4 ± 0.2 $^{c}$ | 5.8 ± 0.1 $^{b}$ | 5.0 ± 0.1 $^{d}$ | 4.7 ± 0.1 $^{e}$ | 5.4 ± 0.1 $^{c}$ |
| **b*** | 21.5 ± 0.1 $^{a}$ | 19.3 ± 0.8 $^{b}$ | 17.3 ± 0.0 $^{d,e}$ | 18.0 ± 0.6 $^{c,d}$ | 16.2 ± 0.4 $^{f}$ | 17.0 ± 0.4 $^{e}$ | 19.2 ± 0.1 $^{b}$ | 18.7 ± 0.1 $^{b,c}$ |

**Highlights:**

- Chemical composition of bread made with ancient, heritage and modern grains is similar.
- In vitro digestion allows cell supplementation with bioaccessible food fraction.
- Biological effect of bread made with ancient, heritage and modern grains is different.
- Ancient grains have protective effects in HepG2 cells.




Graphics Abstract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Supplementation
Analyses
hours
-24
-22
-20
-18
-16
-14
-12
-10
-8
-6
-4
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
B
Cell seeding
Supplementation
Analyses
hours
-24
-22
-20
-18
-16
-14
-12
-10
-8
-6
-4
-2
0
2
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6
8
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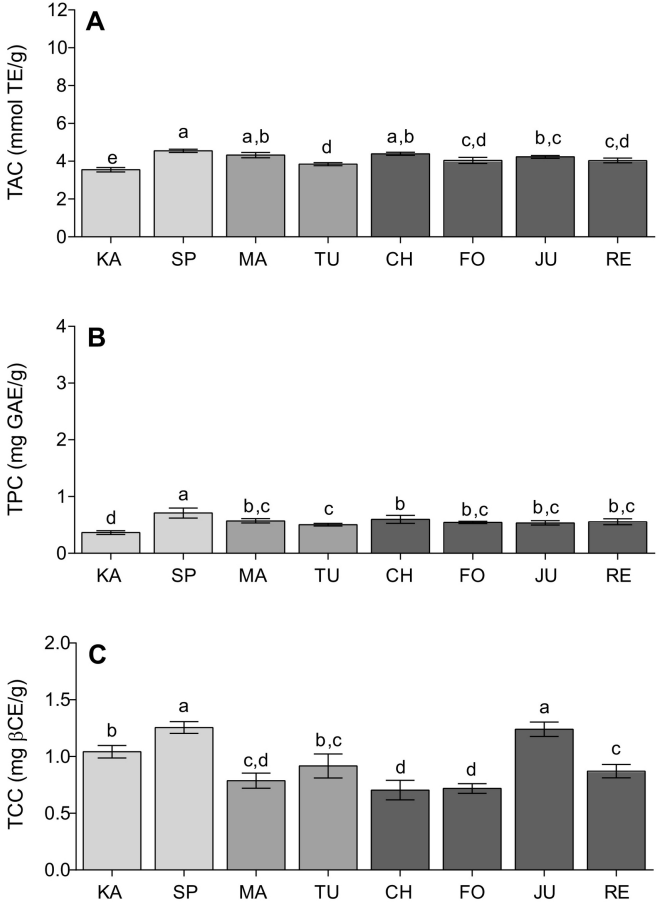
12
14
16
18
20
22
24
26
Change to fresh, not supplemented medium containing the inflammatory mix

Figure 1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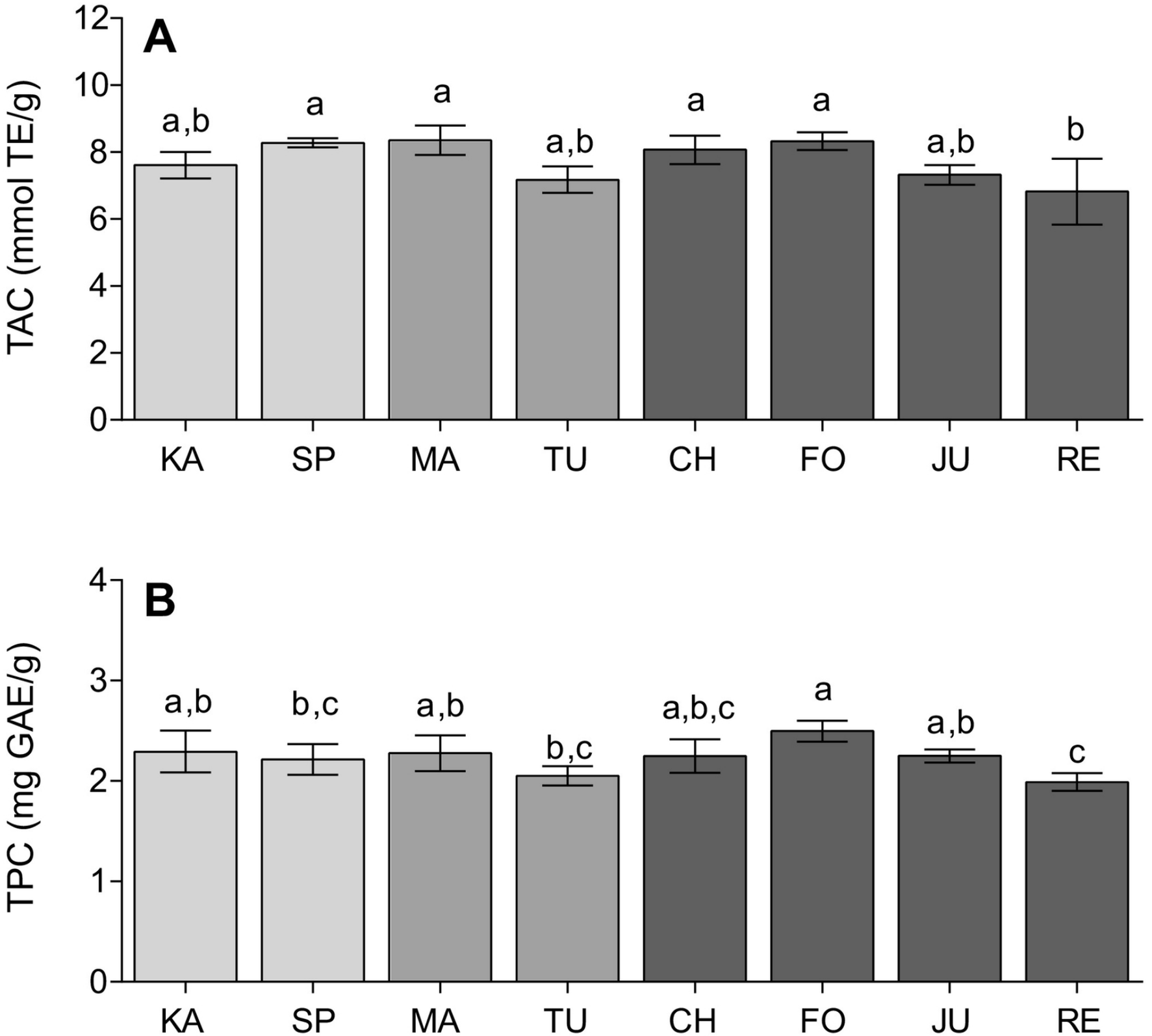
10
8
6
4
2
0
e
a
a,b
d
a,b
c,d
b,c
c,d
KA
SP
MA
TU
CH
FO
JU
RE
B
TPC (mg GAE/g)
4
3
2
1
0
d
a
b,c
c
b
b,c
b,c
b,c
KA
SP
MA
TU
CH
FO
JU
RE
C
TCC (mg βCE/g)
2.0
1.5
1.0
0.5
0.0
b
a
c,d
b,c
d
d
a
c
KA
SP
MA
TU
CH
FO
JU
RE

Figure 2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TAC (mmol TE/g)
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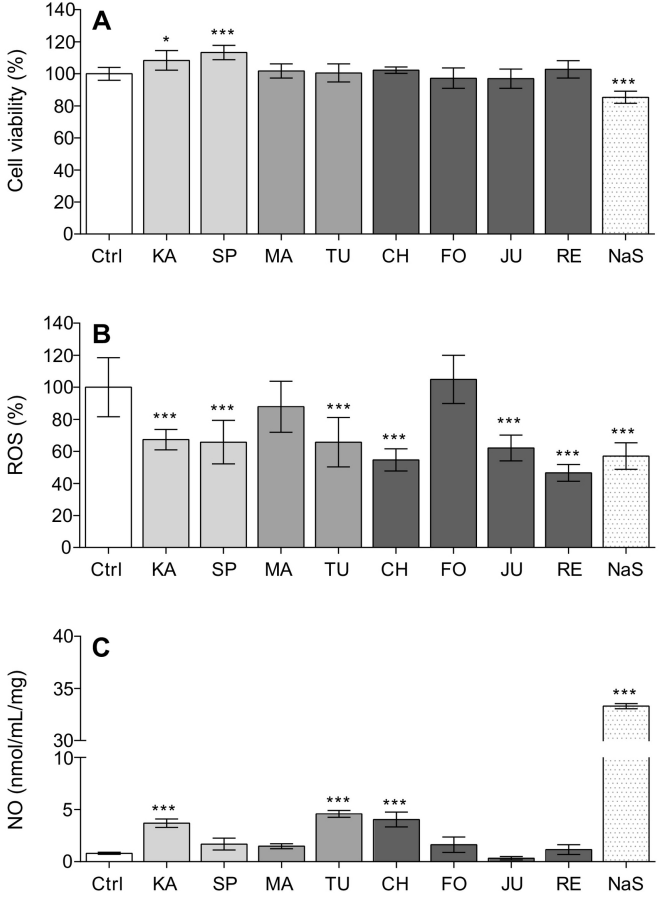
10
8
6
4
2
0
a,b
a
a
a,b
a
a
a,b
b
KA
SP
MA
TU
CH
FO
JU
RE
B
TPC (mg GAE/g)
4
3
2
1
0
a,b
b,c
a,b
b,c
a,b,c
a
a,b
c
KA
SP
MA
TU
CH
FO
JU
RE

Figure 3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Cell viability (%)
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120
100
80
60
40
20
0
*
***
***
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
B
ROS (%)
140
120
100
80
60
40
20
0
***
***
***
***
***
***
***
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
C
NO (nmol/mL/mg)
40
35
30
10
5
0
***
***
***
***
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS

Figure 4

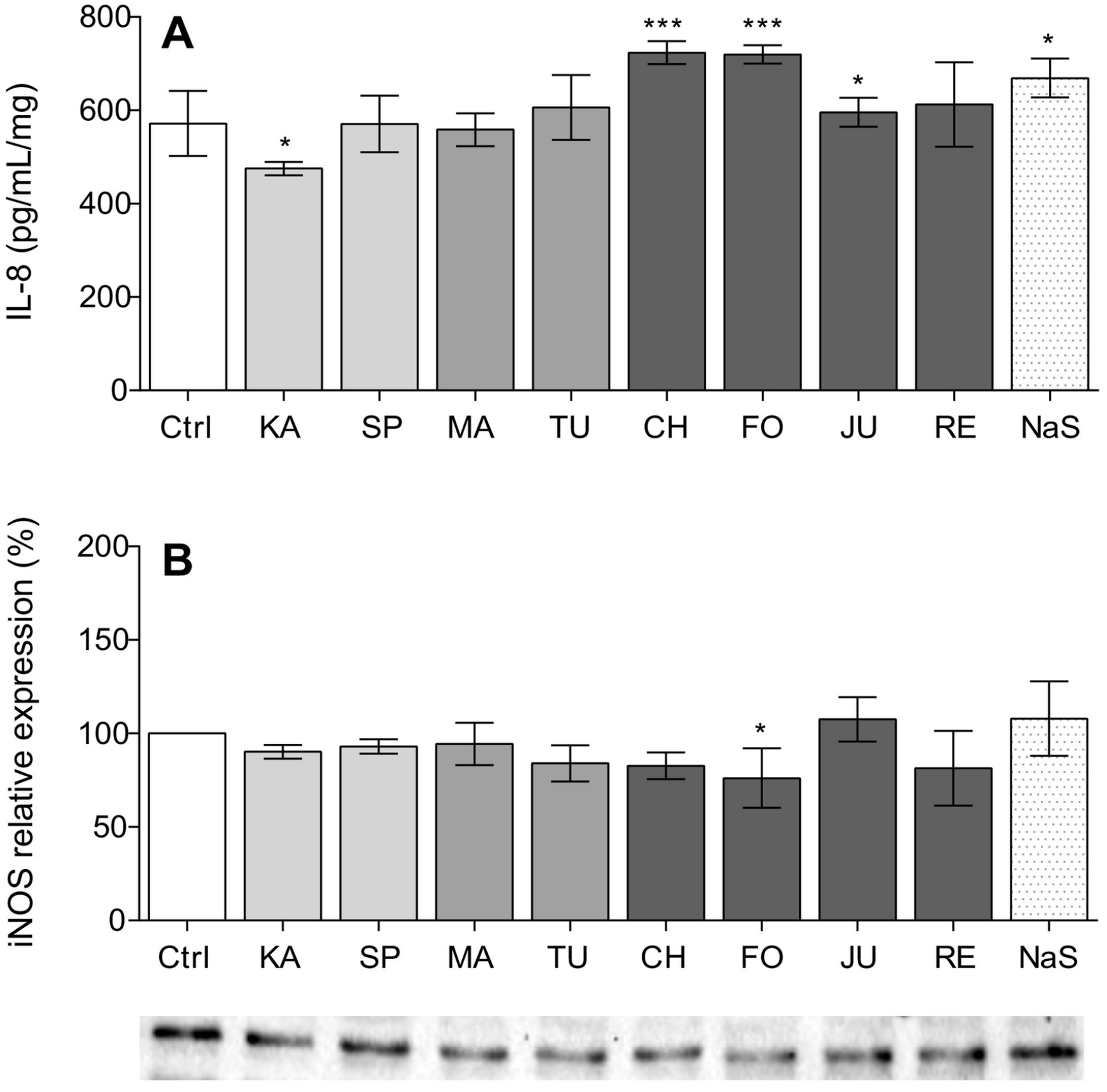
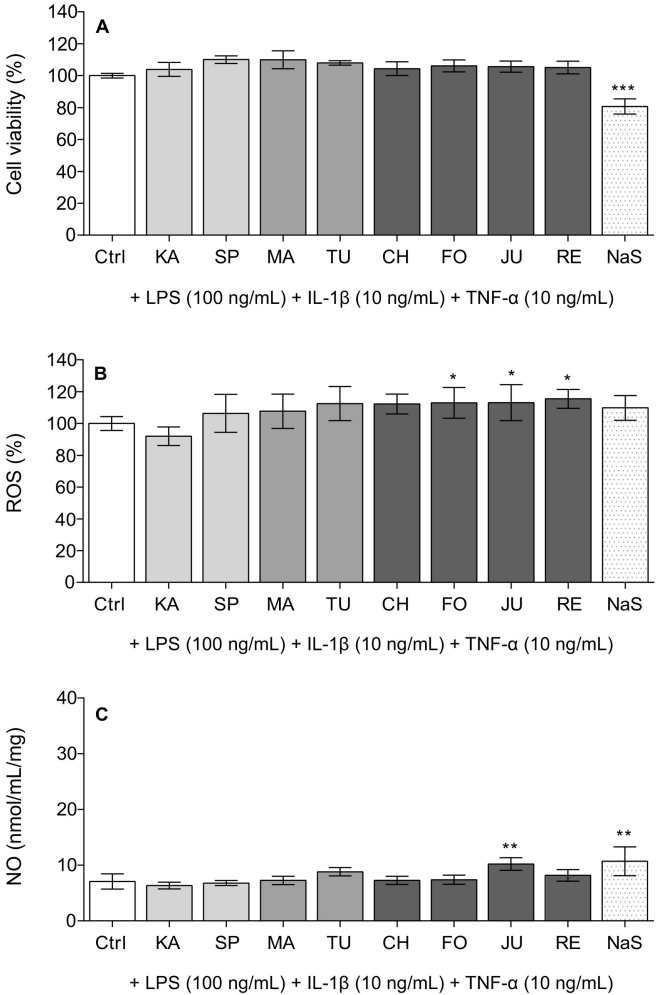

A
IL-8 (pg/mL/mg)
800
600
400
200
0
*
***
***
*
*
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
B
iNOS relative expression (%)
200
150
100
50
0
*
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS

Figure 5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Cell viability (%)
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120
100
80
60
40
20
0
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Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
+ LPS (100 ng/mL) + IL-1β (10 ng/mL) + TNF-α (10 ng/mL)
B
ROS (%)
140
120
100
80
60
40
20
0
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Ctrl
KA
SP
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FO
JU
RE
NaS
+ LPS (100 ng/mL) + IL-1β (10 ng/mL) + TNF-α (10 ng/mL)
C
NO (nmol/mL/mg)
40
30
20
10
0
**
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Ctrl
KA
SP
MA
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CH
FO
JU
RE
NaS
+ LPS (100 ng/mL) + IL-1β (10 ng/mL) + TNF-α (10 ng/mL)

Figure 6

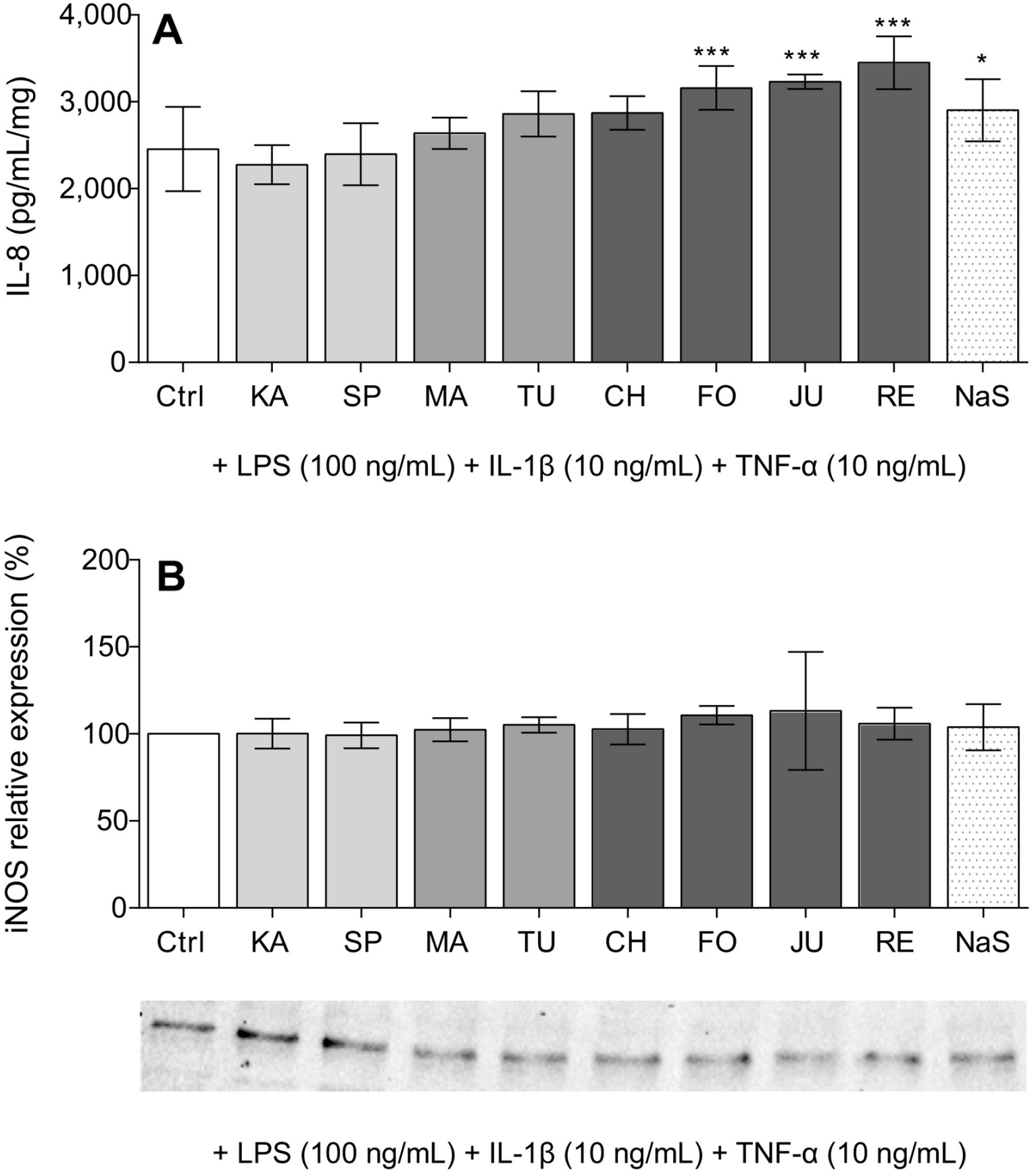
A
IL-8 (pg/mL/mg)
4,000
3,000
2,000
1,000
0
***
***
***
*
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
+ LPS (100 ng/mL) + IL-1β (10 ng/mL) + TNF-α (10 ng/mL)
B
iNOS relative expression (%)
200
150
100
50
0
Ctrl
KA
SP
MA
TU
CH
FO
JU
RE
NaS
+ LPS (100 ng/mL) + IL-1β (10 ng/mL) + TNF-α (10 ng/mL)

Figure 7