



Antioxidative and anti-inflammatory effect of *in vitro* digested cookies baked using different types of flours and fermentation methods



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ABSTRACT

There is an increased amount of evidence showing that consumption of whole grains and whole-grain-based products is associated with a reduction of the risk of developing many diseases, due mainly to the anti-inflammatory and antioxidative effects of their components.

In this study, cookies, baked using different types of flours and fermentation methods, were digested *in vitro* and supplemented to cultured liver cells. Three different flours (ancient KAMUT® khorasan wheat grown in North America, ancient khorasan wheat grown in Italy, and modern durum wheat) and two different types of fermentation (standard and lactic fermentation) were used. This experimental design allowed us to supplement cells with a real food part of the human diet, and to consider possible differences related to the food matrix (types of flour) and processing (methods of fermentation). Cells were supplemented with the bioaccessible fractions derived from cookies *in vitro* digestion. Although results herein reported highlight the antioxidant and anti-inflammatory effect of all the supplementations, cookies made with khorasan flours appeared the most effective, particularly when the ancient grain was grown in North America under the KAMUT® brand. In light of the attempts to produce healthier food, this study underlines the importance of the type of grain to obtain baked products with an increased nutritional and functional value.

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1. Introduction

Besides being sources of dietary fibers whole grain (WG) flours are rich in health-beneficial phytochemicals, including trace minerals and phenolic compounds (Okarter & Liu, 2010), that are concentrated in

the outer layers of the grain. There is much research that suggests the inclusion of whole cereal flours, or mixtures of different grains or their fractions, to increase the nutritional value of products based on refined wheat flour (Sanz-Penella & Haros, 2014). Epidemiological studies confirm that high WG intake protects against cancer, cardiovascular disease, diabetes, and obesity (Slavin, 2003).

The concentration of WG bioactive components has been reported to be higher in ancient crops and/or minor cereals (e.g. KAMUT® khorasan wheat, barley, spelt, and rye), thus increasing the interest on the use of ancient grains because of their better health-related composition (Wijngaard & Arendt, 2006).

In previous studies we demonstrated that compared to modern wheat the ancient KAMUT® khorasan grain is more effective in reducing both oxidative damage and inflammatory status in rats (Benedetti et al., 2012; Carnevali et al., 2014; Gianotti et al., 2011). KAMUT® is a registered trademark associated with the special wheat variety *Triticum turgidum* spp. *turanicum*, also known as khorasan wheat. Khorasan wheat is genetically similar to modern durum wheat with origins that are claimed to be much older. Any khorasan wheat sold under the KAMUT® brand must follow several quality specifications related both to nutritional characteristics and growing conditions (Quinn, 1999). In particular the grain must be organically grown and all seeds used must be unmodified from the original seed stock first used under the

Abbreviations: AGEs, advanced glycation end products; β-CE, β-carotene equivalents; CFU, colony-forming unit; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; DUR_L, cookies made with Italian whole-grain durum wheat fermented using *S. cerevisiae* and LAB; DUR_S, cookies made with Italian whole grain durum wheat fermented using *S. cerevisiae*; EBSS, Earle's balanced salt solution; fwb, flour weight basis; GAE, gallic acid equivalent; GSH, reduced glutathione; IL-8, interleukin-8; IL-10, interleukin-10; KAM_L, cookies made with American KAMUT® khorasan wheat fermented using *S. cerevisiae* and LAB; KAM_S, cookies made with American KAMUT® khorasan wheat fermented using *S. cerevisiae*; KHO_L, cookies made with Italian khorasan wheat fermented using *S. cerevisiae* and LAB; KHO_S, cookies made with Italian khorasan wheat fermented using *S. cerevisiae*; LAB, lactic acid bacteria; LPS, lipopolysaccharides; MRPs, Maillard reaction products; MRS, Man Rogosa Sharpe; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RFU, fluorescence units; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; Se, selenium; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TC, total carotenoids; TE, Trolox equivalents; TP, total polyphenols; HSD, honestly significant difference; US, unsupplemented control cells; WG, whole grain; YPD, yeast extract peptone.

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KAMUT® brand. Additionally, the grain must have a protein content of between 12 and 18% and must contain between 400 and 1000 ppb selenium.

The aim of the present work is to further assess the anti-inflammatory and antioxidant properties of WG KAMUT® khorasan and WG Italian khorasan flours, and to compare them to WG Italian modern durum wheat flour. As reported above, to be sold under the trademark KAMUT®, khorasan wheat must meet specific standards that so far are consistently satisfied only in the growing region of North America; since the level of phytochemicals in plants is strongly affected by agronomic and environmental factors that could therefore have a significant impact on the protective activity of edible plants (Danesi, Valli, Elementi, & D'Antuono, 2014), khorasan wheat grown in Italy and that grown under the KAMUT® brand in North America were compared.

The different flours were used as main ingredients to make cookies, a popular foodstuff consumed by a wide range of populations due to their varied taste, long shelf-life and relatively low cost (Vitali, Dragojević, & Šebečić, 2009). The use of these flours to produce a common food allowed the study of the so-called “food matrix effect”, i.e. the possible impact of the different food components and of the food processing on the overall effect of the putative functional ingredient. To investigate differences related to the fermentation process, cookies were fermented using *Saccharomyces cerevisiae* alone (as standard) or *S. cerevisiae* and lactic acid bacteria (LAB) known as sourdough fermentation. Indeed, it has been shown that cereal fermentation by yeast and LAB, besides an improvement of texture and shelf life, can positively affect food nutritional quality retarding starch digestibility, decreasing the glycemic response, improving the properties and the bioaccessibility of the dietary fiber complex and phytochemicals, and increasing the uptake of minerals (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Poutanen, Flander, & Katina, 2009).

Experiments were performed supplementing cultured liver cells with the bioaccessible fractions derived from cookies *in vitro* digestion, in order to get closer to the *in vivo* situation and to partially fill the gap between *in vivo* and *in vitro* studies.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were from Lonza (Milan, Italy). All other chemicals were from Sigma-Aldrich (Milan, Italy). All chemicals and solvents were of the highest analytical grade.

Flours: Italian whole grain khorasan and Italian whole grain modern durum wheat (cv. Claudio) flours were obtained from the Department of Agricultural Sciences, University of Bologna (Italy), while North American whole grain KAMUT® khorasan flour was a kind gift of Kamut Enterprises of Europe (Oudenaarde, Belgium). All grains used to produce the flours were organically grown.

Other ingredients: butter, eggs, sugar, and salt were purchased at local markets. Strains: *Lactobacillus plantarum* (98a), *Lactobacillus sanfranciscensis* (BB12), *Lactobacillus brevis* (3BHI), and *S. cerevisiae* (LBS) strains belong to the Department of Agricultural and Food Science and Technology (DISTAL) of the University of Bologna (Italy).

2.2. Analysis on flours

Moisture, total nitrogen, carbohydrates, lipids, fibers and ashes were evaluated according to Baldini et al. (1996). Selenium concentration in the different flours was determined by inductively coupled plasma-atomic emission spectrometry (Navarro-Blasco & Alvarez-Galindo, 2004). Total carotenoids were determined using the method described by Koca, Burdurlu, and Karadeniz (2007) with some modifications. Briefly, 1 g of flour was mixed with 5 mL of hexane-

acetone (70:30, v/v), vortexed twice at high speed, sonicated, and centrifuged at 4500 × g for 20 min. The absorbance of the supernatants was measured at 450 nm and compared to the concentration–response curve of β-carotene standard. Results were expressed as micrograms of β-carotene equivalents (β-CE)/100 g.

To evaluate the total antioxidant capacity (TAC) and the total polyphenols (TP) content 1 g of each flour was extracted according to Danesi et al. (2013) with a final volume of 6 mL ethanol/water (70:30) acidified with 0.1% HCl. TP content was determined using Folin–Ciocalteu's method (Singleton & Rossi, 1965), adapted to a 96-well plate assay according to Dicko et al. (2002). Results were expressed as mg gallic acid equivalent (GAE)/100 g. TAC was measured using the method of Re et al. (1999) and the values obtained were expressed as μmol of Trolox equivalents (TE)/100 g.

2.3. Strain growth media

LAB strains were grown separately in the Man Rogosa Sharpe (MRS) broth (Oxoid, Milan, Italy) at 37 °C for 24 h and the *S. cerevisiae* strain was grown in the yeast extract peptone dextrose (YPD) broth at 28 °C for 24 h. The cells have been harvested by centrifugation 4000 × g, for 10 min, and washed twice with sterile water before their use in cookies preparation.

2.4. Cookies preparation: formulation, fermentation and baking process

Six different types of cookies were tested: i. Cookies made with Italian whole grain durum wheat fermented using *S. cerevisiae* (DUR_S); ii. Cookies made with Italian khorasan wheat fermented using *S. cerevisiae* (KHO_S); iii. Cookies made with American KAMUT® khorasan wheat fermented using *S. cerevisiae* (KAM_S); iv. Cookies made with Italian whole-grain durum wheat fermented using sourdough, namely *S. cerevisiae* and LAB (DUR_L); v. Cookies made with Italian khorasan wheat fermented using sourdough, namely *S. cerevisiae* and LAB (KHO_L); vi. Cookies made with American KAMUT® khorasan wheat fermented using sourdough, namely *S. cerevisiae* and LAB (KAM_L).

Specifically, for sourdough fermentation a 10% (fwb) mixed-strain containing *Lb. plantarum* 98a, *Lb. sanfranciscensis* BB12, *Lb. brevis* 3BHI, was added to the dough and fermented at 32 °C for 24 h. To prepare the cookies, 3% (fwb) or 14% (fwb) of *S. cerevisiae* LBS was added to the final dough for 1.5 h of leavening at 32 °C. The inoculums level in dough was approximately 10⁹ CFU/g for LAB and 10⁷ CFU/g for *S. cerevisiae*.

All cookies were made according to the same recipe (Table 1), keeping constant the amount of all ingredients but the mixture of microorganisms used for fermentation, then cookies were baked in oven at the same temperature (175 °C) for the same time (10 min). Each kind of cookie was prepared twice in two different days, and the two preparations were mixed in order to consider the variability that can derive from these preliminary phases.

Table 1
Cookie recipe, modified by Drewnowski et al. (1998).

Ingredients	DUR _L , KHO _L , KAM _L		DUR _S , KHO _S , KAM _S	
	Absolute quantities	Relative quantities	Absolute quantities	Relative quantities
Flour	140 g	48.65 w/w	140 g	48.65 w/w
Butter	56.5 g	19.64 w/w	56.5 g	19.64 w/w
Eggs	44 g	15.29 w/w	44 g	15.29 w/w
Sugar	24.25 g	8.43 w/w	24.25 g	8.43 w/w
Salt	3 g	1.04 w/w	3 g	1.04 w/w
Mix of LAB	15 mL	5.21 v/v	–	–
<i>S. cerevisiae</i>	5 mL	1.74 v/v	20 mL	6.95 v/v

2.5. *In vitro* digestion

Cookies were digested *in vitro* according to Bordonni et al. (2011) with slight modifications. Briefly, the *in vitro* digestion was simulated inside a 100 mL shaking flask at 37 °C. For 1 g of biscuit dry matter, 2: 4: 4 mL of a buffer solution (120 mM NaCl, 5 mM KCl, 6 mM CaCl₂ – pH 6.9) was added to resemble saliva, gastric juice and duodenal juice respectively. The absolute volumes of the digestive juices were scaled to the content of food used. Mastication and oral digestion were simulated by adding buffer solution with 90 U/mL α -amylase and grinding the biscuits with mortar and pestle for 5 min. Then, buffer solution was added and the pH was decreased to 2.0 by 37% HCl. Gastric digestion started with the addition of pepsin (3 mg/mL final concentration). After 60 min of incubation, buffer solution was added and pH increased to 5 with 1.5 M NaHCO₃ to stop peptic digestion. Duodenal digestion, followed for 180 min, started with the addition of pancreatin (0.4 mg/mL final concentration), bile (2.4 mg/mL final concentration), and a pH adjustment to 6.5. Digestion was performed three times for each kind of cookie, and the resulting final digested solutions were mixed and frozen, in order to supplement cells with the same solution in each experiment.

Prior to supplementation, the digested mix was centrifuged at 4000 \times g for 5 min, the upper lipid layer was removed and the lower aqueous phase was centrifuged again at 21,000 \times g for 20 min. The supernatant was filtered with 0.2 μ m membranes and an aliquot was sequentially ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (EMD Millipore; Billerica, MA, US). This permitted the cells to be supplemented with a mixture of compounds small enough to be absorbable through the intestinal mucosa (bio-accessible fraction).

2.6. HepG2 cells culture and supplementation

HepG2 cells, a human hepatoma cell line considered a good model to study *in vitro* cytotoxic agents (Mersch-Sundermann, Knasmüller, Wu, Darroudi, & Kassie, 2004), were chosen as model system given that the liver is the organ mainly involved in xenobiotic metabolism (Goya, Delgado-Andrade, Rufián-Henares, Bravo, & Morales, 2007).

HepG2 cells were grown in DMEM with 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, and maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Once a week cells were split 1:20 into a new 75 cm² flask, and culture medium was changed every 48 h.

Cells were seeded in 6-well or 12-well plates at the concentration of 1 \times 10⁶ cells/mL. Cell counting was carried out using the TC20™ Automated Cell Counter (Bio-Rad Laboratories; Hercules, CA, US). After 24 h (75–80% confluence) cells were incubated with serum-free DMEM containing the different digested mixtures. The concentration of the digested mixtures to be used for cell supplementation was determined by preliminary experiments by assessing the possible cytotoxicity (data shown in the Supplementary material); the 50 μ L/mL concentration didn't cause any cytotoxic effect in basal condition and was therefore used for experiments. Unsupplemented control cells (US) received a corresponding amount of sterile water.

In experiments aimed at investigating the protection against an oxidative damage, 24 h after supplementation cells were exposed for 1 h to 0.4 mM H₂O₂ in Earle's balanced salt solution (EBSS) (Di Nunzio, Toselli, Verardo, Caboni, & Bordonni, 2013). Some US cells were not stressed and received EBSS without H₂O₂. After 1 h EBSS was removed, centrifuged at 400 \times g for 3 min, and used for the thiobarbituric acid reactive substances (TBARS) assay.

In experiments aimed at investigating the anti-inflammatory activity, 6 h after supplementation the media were changed and cells were incubated with Roswell Park Memorial Institute (RPMI)-1640 medium modified without phenol red containing 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM glutamine, and the 50 μ L/mL of the different solutions of digested cookies or water which was used as a control. To induce an inflammatory stimulus some cells also received 100 ng/mL of

lipopolysaccharides (LPS), known to be a strong stimulator of inflammatory response (Hong, Seo, Lee, & Choi, 2004). After 18 h the medium was removed and maintained at – 20 °C until cytokines were quantified.

2.7. Cell viability

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, according to Di Nunzio et al. (2013). Results were expressed as percentage of the value obtained in non-stressed, US cells.

2.8. Protection against the oxidative damage

To evaluate the possible counteraction of the oxidative damage, TBARS concentration in the EBSS medium and intracellular ROS levels were assessed as described in details by Valli et al. (2012). Fluorescence intensity was detected (λ_{ex} = 535 nm, λ_{em} = 595 nm; λ_{ex} = 485 nm, λ_{em} = 535 nm for TBARS and ROS, respectively) using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland). TBARS levels were expressed as relative fluorescence units (RFU) and normalized for milligrams of proteins in each well; whereas ROS concentrations, normalized for protein content, were stated as percent of the value found in non-stressed US cells. Protein content was determined according to Bradford (1976) using bovine serum albumin as standard.

2.9. Evaluation of the antioxidant defenses

The potential modulation of antioxidant defenses by the different digested cookies was assessed by measuring the cytosolic TAC and the thiolic content. Cells were washed twice with cold DPBS, lysed with cold Nonidet P-40 (0.25% in DPBS), incubated for 30 min on ice while shaking, and centrifuged at 14,000 \times g for 15 min.

Cytosolic TAC was measured on the supernatant using the method of Re et al. (1999), and results were normalized for protein content in the sample.

The total thiolic content, considered as GSH, was determined as previously described by Di Nunzio et al. (2013). The obtained values were normalized for protein content in the sample, and expressed as nmol of thiols calculated as GSH/mg protein. Protein content was determined according to Bradford (1976) using bovine serum albumin as standard.

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 the media in both basal condition and after cell treatment with LPS using the Multi-Analyte ELISArray Kit (Qiagen; Hilden, Germany) quantitative sandwich immunoassay. Results were normalized for cell protein content (Bradford, 1976), and expressed as percentage of non-stressed US.

2.11. Statistical analysis

Statistical comparison was performed by analysis of variance (one-way ANOVA for the flours nutritional composition and three-way ANOVA for cell culture analysis). Tukey's honestly significant difference (HSD) test was used as post-hoc test. Different letters indicate statistical significance (at least P < 0.05).

3. Results

3.1. Flours nutritional composition

The nutritional composition of the different flours is presented in Table 2. KAMUT® flour showed the lowest content in water and the highest content in total nitrogen, while no significant differences in

Table 2Nutritional composition of the different flours used for cookie preparation. Data are means \pm SD (n = 2 to 4).

	Italian durum wheat flour	Italian khorasan flour	North American KAMUT® flour
Water (g/100 g)	12.10 \pm 0.61 ^a	12.04 \pm 0.60 ^a	10.36 \pm 0.52 ^b
Total nitrogen (g/100 g)	9.99 \pm 0.50 ^b	10.74 \pm 0.54 ^b	12.83 \pm 0.64 ^a
Available carbohydrates (g/100 g)	63.50 \pm 16.90 ^a	65.04 \pm 16.9 ^a	63.61 \pm 16.54 ^a
Lipids (g/100 g)	1.61 \pm 0.16 ^a	1.56 \pm 0.16 ^a	1.83 \pm 0.18 ^a
Fibers (g/100 g)	11.20 \pm 1.12 ^a	8.87 \pm 0.89 ^a	9.89 \pm 0.99 ^a
Ash (g/100 g)	1.61 \pm 0.24 ^a	1.75 \pm 0.26 ^a	1.47 \pm 0.22 ^a

Different letters in the same row indicate statistically significant differences (at least $P < 0.05$).

available carbohydrates, lipids, fibers and ash content were detected among the different flours.

Since it is known that selenium (Se) content in foods greatly depends on its concentration in the soil (Stadlober, Sager, & Irgolic, 2001), and Se concentration in North American soil is reported to be higher than in Italy (Combs, 2001), Se concentration was also analyzed in the different flours. Results confirmed a higher Se concentration in the North American KAMUT® flour than in flours from grains grown in Italy (Table 3).

In addition, since previous studies showed that both genotype and growing conditions may impact on the antioxidant properties and content of bioactive molecules such as phenolic compounds and carotenoids of wheat (Lu et al., 2015; Zhou, Su, & Yu, 2004), the total carotenoids (TC), the total polyphenols (TP) content, and the total antioxidant capacity (TAC) were also determined in the flours (Table 3). The KAMUT® flour was found to be the richest in TC and TP, and showed the highest TAC as well. Comparing flours obtained from wheat grown in Italy, the khorasan flour showed higher TC and TP content than the durum wheat flour.

3.2. Antioxidative activity

Compared to US cells in basal condition, the exposure to H₂O₂ caused a reduction in cell viability in all cells except those supplemented with KAMUT® digested cookies (Fig. 1). In stressed condition, cell viability was anyway higher in supplemented cells than in US ones.

In all cells the exposure to H₂O₂ greatly increased TBARS concentration in the medium. Among stressed cells this increase was higher in unsupplemented than in supplemented ones, KAM_S and KAM_L showed the lowest increase (Fig. 2A).

Upon H₂O₂ treatment, ROS concentration significantly increased in US cells, while it did not change or even decreased in supplemented ones (Fig. 2B).

H₂O₂ exposure caused a significant decrease in TAC in only US cells as well. In stressed cells, a higher TAC was evidenced in KAM_S and KAM_L supplemented cells than in unsupplemented ones (Fig. 3A).

In US cells, the total thiolic intracellular content was not modified by the oxidative stimulus; however, upon H₂O₂ exposure it increased in all supplemented cells, regardless the type of supplementation (Fig. 3B).

3.3. Cytokine secretion

To evaluate the potential anti-inflammatory effect of the different digested cookies, the levels of a pro-inflammatory and an anti-inflammatory (interleukin 8, IL-8, and interleukin 10, IL-10,

respectively) cytokine were estimated in the media in both basal condition and after cell treatment with LPS.

In basal condition, all supplementation except DUR_S induced a significant decrease in the production of pro-inflammatory IL-8. This decrease was particularly evident in cells supplemented with cookies prepared using sourdough fermentation, regardless the type of flour (Fig. 4A). In a complementary way the secretion of the anti-inflammatory IL-10 increased in all supplemented cells compared to US ones, except the one receiving the DUR_S digested cookies (Fig. 4B). Variance analysis revealed evidence of interaction between grains origin and fermentation process ($P < 0.001$; data not shown) in IL-10 secretion.

Upon LPS exposure, a significant increase of the pro-inflammatory IL-8 secretion was observed in US cells; IL-8 production was lower in all supplemented cells than in unsupplemented ones (Fig. 5A). Compared to US cells in basal condition, KAM cells evidenced a lower IL-8 secretion, independently of the type of fermentation used for cookies preparation.

The production of anti-inflammatory cytokines represents the cellular physiological response to an inflammatory stimulus (Schottelius, Mayo, Sartor, & Baldwin, 1999). Actually, after 18 h exposure to LPS, IL-10 secretion increased in both unsupplemented and supplemented cells but the one receiving the DUR_L digested cookies. The highest IL-10 secretion was evidenced in cells supplemented with Italian khorasan and North American KAMUT® khorasan cookies (Fig. 5B).

4. Discussion

Cereals are the dominant crops in world agriculture, and they are the first source of calories and protein to the diets of humans. The benefits of WG higher intake could be ascribed not only to fiber, but also to a plethora of phytochemicals that have been reported to exert both antioxidant and anti-inflammatory effects (Makni et al., 2011; Mateo Anson, Havenaar, Bast, & Haenen, 2010).

In this study both the antioxidant and anti-inflammatory effects of different wholegrain flours have been evaluated in cultured cells. Flours were used to make cookies, in order to supplement cells with real foods that are part of the human diet rather than using raw materials which are not normally used as food. This also permitted the consideration of the effects of the food matrix using several different types of flours and two different types of fermentation (standard and sourdough fermentation).

Furthermore, cells were not supplemented with the food extracts, but with the bioaccessible fractions derived from cookies subjected to *in vitro* digestion in order to evaluate possible differences resulting from the enzymatic breakdown of organic macromolecules, which more closely mimics the *in vivo* situation.

Table 3Selenium, total carotenoids, total polyphenols and total antioxidant capacity of the different flours. Data are means \pm SD (n = 2 to 4).

	Italian durum wheat flour	Italian khorasan flour	North American KAMUT® flour
Selenium (μ g/100 g)	7.00 \pm 3.00 ^b	5.00 \pm 2.00 ^b	70.00 \pm 20.00 ^a
Total carotenoids (μ g β -CE/100 g)	234.22 \pm 5.62 ^c	266.40 \pm 8.29 ^b	297.66 \pm 13.80 ^a
Total polyphenols (mg GAE/100 g)	109.88 \pm 5.62 ^c	130.75 \pm 8.07 ^b	149.48 \pm 8.87 ^a
Total antioxidant capacity (μ mol TE/100 g)	556.76 \pm 4.43 ^b	575.60 \pm 26.33 ^b	618.73 \pm 2.97 ^a

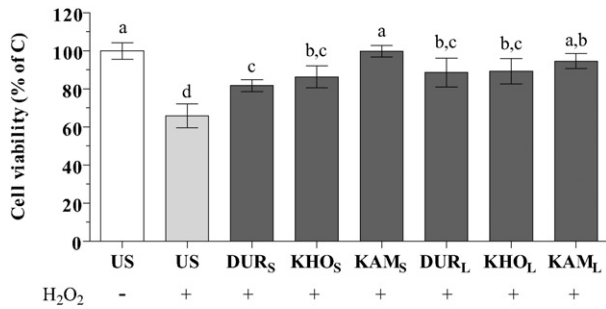


Fig. 1. Cell viability in control and supplemented cells after the oxidative damage. Data are means \pm SD of at least 6 samples derived from 3 independent cell cultures.

All tests used to evaluate the protective effect of the different supplementations against an induced oxidative stress (H₂O₂ exposure) confirmed the effectiveness of all the digested cookies, although the ones made with KAMUT® khorasan flour better preserved cells, particularly from the increase in TBARS and the decrease in viability. This last observation is in agreement with previous studies in rats (Benedetti et al., 2012; Gianotti et al., 2011) and humans (Sofi et al., 2013).

To evaluate the possible effect of the different cookies on cell cytokine secretion, IL-8 and IL-10 were chosen as markers since HepG2 cells are capable of producing them in response to specific stimulation. Moreover, 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), whereas IL-8 is a pro-inflammatory molecule also inducing cytotoxic effects (Makni et al., 2011).

In basal condition, the lower IL-8 and the higher IL-10 secretion observed in supplemented cells than in unsupplemented ones suggest an anti-inflammatory action of the components in the flours.

Sourdough fermentation based on LAB appeared to significantly improve the positive effect of the digested cookies on cytokine secretion. This is in agreement with Wang, Wu, and Shyu (2014) who observed that cereals fermented by *Lb. plantarum* significantly inhibited the production of LPS-induced intracellular reactive oxygen species (ROS)

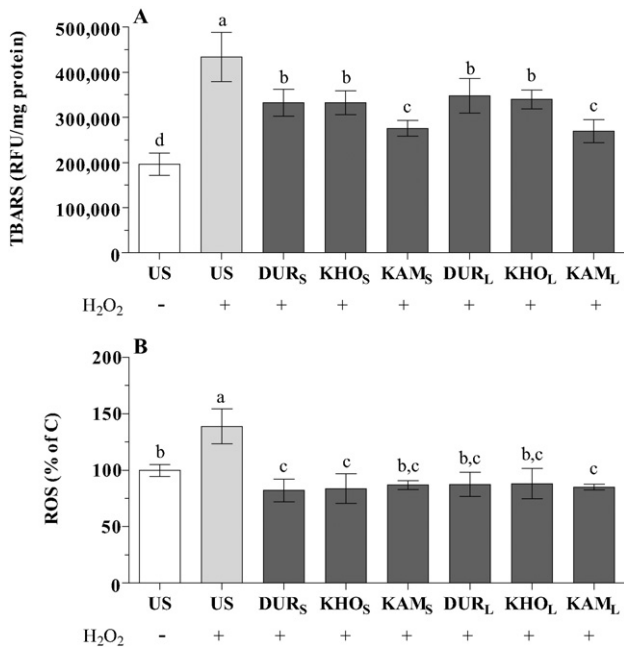


Fig. 2. TBARS and ROS concentration in control and supplemented cells after the oxidative damage. TBARS: panel A; ROS: panel B. Data are means \pm SD of at least 6 samples derived from 3 independent cell cultures.

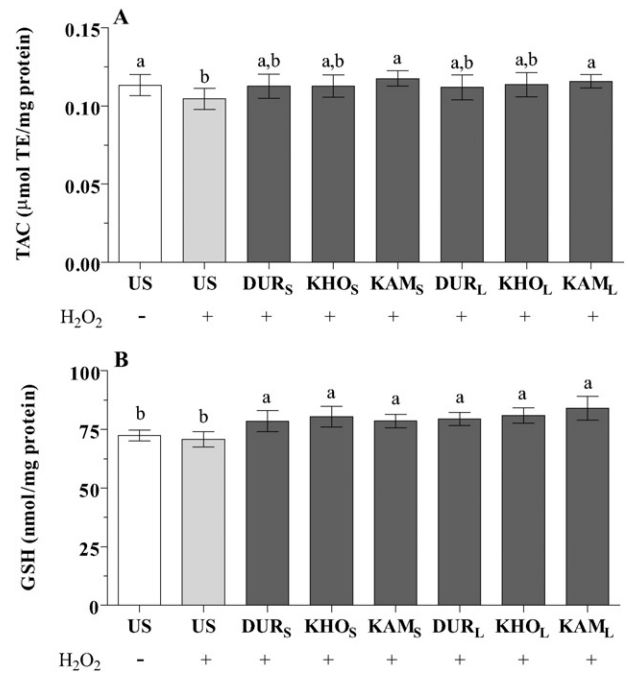


Fig. 3. Cytosolic TAC and GSH content in control and supplemented cells after the oxidative damage. TAC: panel A; GSH: panel B. Data are means \pm SD of at least 6 samples derived from 3 independent cell cultures.

without creating obvious cytotoxic effects in the macrophage cells RAW 264.7. The ability of fermentation to improve antioxidant activity is primarily ascribed to an increase in the amount of phenolic compounds and flavonoids as a result of a microbial hydrolysis reaction. Moreover, fermentation induces the structural breakdown of plant cell walls, leading to the liberation or synthesis of various antioxidant

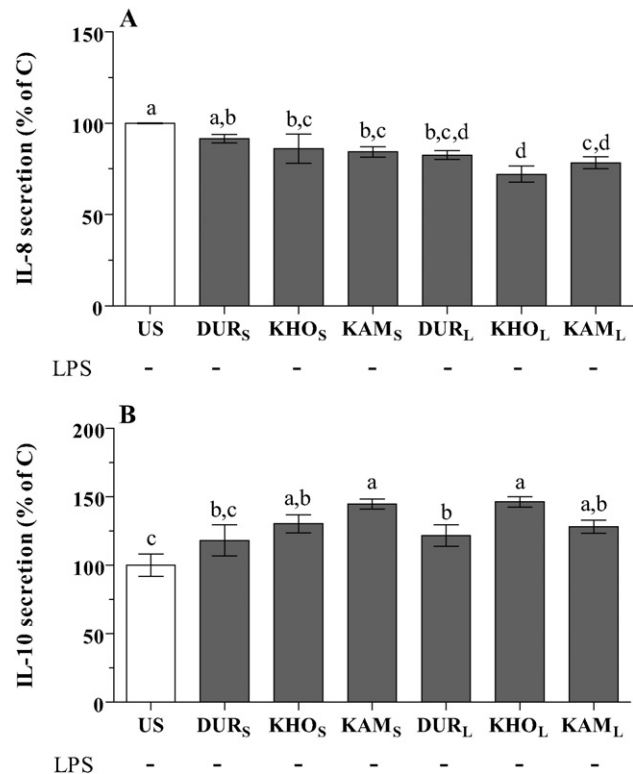


Fig. 4. IL8 and IL-10 secretion in basal conditions. IL-8: panel A; IL-10: panel B. Data are means \pm SD of at least 6 samples derived from 3 independent cell cultures.

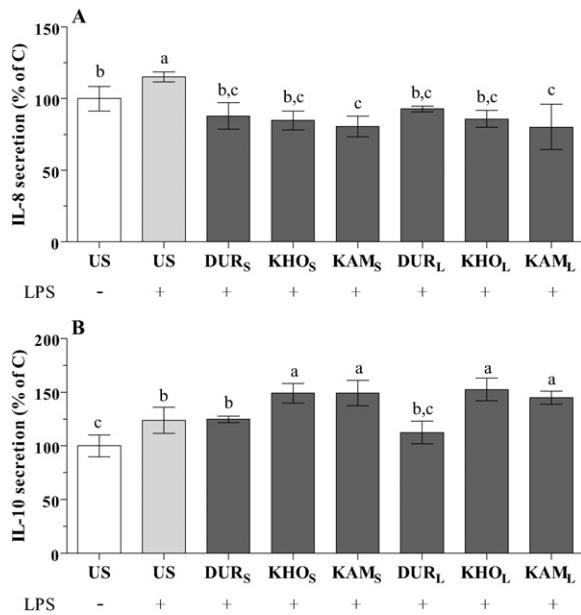


Fig. 5. IL-8 and IL-10 secretion after the pro-inflammatory stimulus. IL-8: panel A; IL-10: panel B. Data are means \pm SD of at least 6 samples derived from 3 independent cell cultures.

compounds that can act as free radical terminators, metal chelators, singlet oxygen quenchers, or hydrogen donors to radicals (Hur, Lee, Kim, Choi, & Kim, 2014). Metabolism of sourdough microbiota and the activity of cereal enzymes are interdependent. Acidification, oxygen consumption, and thiols accumulation by microbial metabolism modulate the activity of cereal enzymes; in turn, cereal enzymes provide substrates for bacterial growth (Ganzle, 2014). In different flours the combination of specific cereal enzymes and the metabolism of lactic acid bacteria could produce a different conversion of carbohydrates, proteins, phenolic compounds and lipids leading to different overall effects.

The anti-inflammatory effects of cell supplementation with cookies made with Italian khorasan and KAMUT® khorasan flours were even more evident after LPS stimulation. These findings are in agreement with previous *in vivo* results; in fact, in a previous work performed on rats fed with KAMUT® khorasan wheat pasta or modern durum wheat pasta we observed histological modifications in the lymph nodes and spleen, and morphological alterations of the intestinal villi that were suggestive of an anti-inflammatory effect of KAMUT® khorasan components compared to an inflammatory effect of modern durum components (Carnevali et al., 2014). Furthermore other studies recently evidenced in humans that a replacement diet with KAMUT® khorasan products is effective in reducing some markers of inflammation (Sofi et al., 2013, 2014).

WG cereal antioxidant and anti-inflammatory properties are mainly ascribed to their phenolic content, and ancient grain varieties were shown to present unique health-promoting phytochemicals in their phenolic profile (Dinelli et al., 2009). Although it is not possible to predict the biological effect of a digested food based on the composition of its ingredients, in the herein reported study the higher TP and TC content detected in KAMUT® khorasan and Italian khorasan flours than in modern durum wheat flour is in agreement with the higher protective effect of the formers than the latter.

The higher antioxidant activity by North American KAMUT® khorasan flour than Italian khorasan flour could be ascribed to the higher content of Se in the former than in the latter. Selenium is an important contributor to the antioxidant potential, since it is a constituent of seleno-enzymes with antioxidant functions (Rayman, 2000). In addition KAMUT® khorasan flour showed the highest TAC, TC and TP content, and this could explain the better effectiveness of cell supplementation with

cookies made with the flour of the ancient khorasan grain cultivated in North America under the KAMUT® brand.

In addition, differences among the different cookies could be related to the Maillard reaction, which is responsible for the characteristic color and taste of baked foods. It has been reported that some Maillard reaction products (MRPs), particularly melanoidins, have beneficial effects as antioxidant and anti-inflammatory factors (Delgado-Andrade, 2014), while advanced glycation end products (AGEs) are considered pro-inflammatory and toxic (Davis, Prasad, Vijayagopal, Juma, & Imrhan, 2014; Liu, Teodorowicz, van Boekel, Wichers, & Hettinga, 2016). As already reported in muffins baked with rice, wheat, corn, oat or barley flour (Soong, Tan, Leong, & Henry, 2014) it is conceivable that the different flours used to bake cookies, as well as the different fermentation process, led to a different production of both MRPs and AGEs, this contributing to the different antioxidant and anti-inflammatory effect.

Overall, cell supplementation with the bioaccessible fraction of digested cookies made with the different flours evidenced protective activities towards oxidative and inflammatory stress, however, the extent of this protection varied from flour to flour (KAMUT® khorasan > Italian khorasan > durum wheat). It is worth noting that all cookies were prepared using WG flours; the higher bioactive content of WG than refined cereals is well known (Onyeneho & Hettiarachchy, 1992; Slavin, 2003), and this could have reduced differences among the studied cereals.

Although the approach used in this study does not allow to evidence exactly which flour components are the protective ones, this must not be considered as a limitation of the study since it allowed us to consider the possible synergism among the different molecules and the importance of some aspects related to the food matrix (*i.e.* different geographical origin of khorasan wheat and different fermentation), both well recognized factors of the overall antioxidant effectiveness (Fardet, 2010) that are ignored in studies evaluating the effect of pure compounds.

5. Conclusions

Considering that most studies regarding the healthy properties of cereals are based on unprocessed or partially processed cereals, and differences in bioactive solubility and/or bioavailability within the digestive tract are not taken into account, the current study represents a further step for the evaluation of different cereals and processing, and highlights the potential differences in health effects of different whole grains grown and processed in various ways. In particular, cookies made with khorasan flours appeared the most protective, especially the KAMUT® khorasan grain ones.

Since attempts are being made to produce healthy and functional food products due to the increased consumers' demand (Ajila, Leelavathi, & Prasada Rao, 2008; Sudha, Vetrmani, & Leelavathi, 2007; Tyagi, Manikantan, Oberoi, & Kaur, 2007; Vitali et al., 2009), results herein reported could represent a good chance to obtain cookies as well as other processed wheat products with an increased nutritional value.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2015.12.010>.

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