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BIOACTIVE COMPONENTS AS INTEGRAL PART OF FOODS AND DIET: EVALUATION OF EFFECTIVENESS AND MECHANISMS OF ACTION

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LIST OF ABBREVIATIONS

ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AGEs: advanced glycation end products; BHT: 3,5-di-tert-4-butylhydroxytoluene; CH: bread made with Choteau grain; CTRL: control; CORN: Corno di Toro pepper; DCFH: 2',7'-dichlorofluorescein; DCFH-DA: 2',7'-dichlorofluorescein diacetate; DMEM: Dulbecco's modified Eagle's medium; DNA: Deoxyribonucleic acid; DPBS: Dulbecco's phosphate-buffered saline; DPPH: 1,1-diphenyl-2-picrylhydrazyl; DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid); EBSS: Earle's balanced salt solution; EDTA: ethylenediaminetetraacetic acid; FO: bread made with Fortuna grain; GAE: gallic acid equivalent; GR: glutathione reductase; GSH: reduced glutathione; IL-1β: interleukin-1β; IL-8: interleukin 8; IL-10: interleukin 10; iNOS: inducible nitric oxide synthase; JU: bread made with Judy grain; KA: bread made with Kamut® grain; LAM: Lamuyo pepper; LDH: lactate dehydrogenase; LPS: lipopolysaccharides; MA: bread made with Marquis grain ; MJ: mandarin juice; MJ20: mandarin juice with homogenization at 20 MPa; MJ20+Tr: mandarin juice with homogenization at 20 MPa in the presence of 10% trehalose; MJ20+Ls: homogenization at 20 MPa in the presence of *Lactobacillus salivarius*; MRP: Maillard reaction products; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: nitric oxide; RFU: relative fluorescence units; ROS: reactive oxygen species; RE: bread made with Redwin grain; RPMI: Roswell Park Memorial Institute; SD: standard deviation; SDS: sodium dodecyl sulfate; SP: bread made with Spelt grain; SS: sodium salicylate; TAA: total antioxidant activity; TAC: total antioxidant capacity; TBA: 2-thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; TCC: total carotenoid content; TCA: trichloroacetic acid; TE: trolox equivalent; TNF- α: tumor necrosis factor α; TPC: total phenolic content; TQ: *talis quails*; TU: bread mead with Turkey red grain ; USx: unsupplemented cells.

PREFACE

It has been well documented that consumption of vegetable foods plays an important role against the onset of chronic diseases such as diabetes, cardiovascular disease and cancer (1-2). These protective effects are generally ascribed to the characteristic nutritional composition of vegetables, which generally contains high amount of unsaturated fatty acids, fiber, vitamins, and minerals. In addition, the presence of various components also known as phytochemicals (3) represent a positive trait of vegetable food.

In vitro and *in vivo* studies suggest that many of these phytochemicals have biological activities, and exhibit the capacity to modulate one or more metabolic processes, which results in the promotion of better health (4). These properties may be linked to their antioxidant and anti-inflammatory activity (5-6).

Each plant food has a different content and profile of bioactive compounds, with specific chemical structure, bioavailability, metabolism and excretion. In addition, several factors as genetics, agronomic conditions and processing are known to affect the content of these compounds (7).

The overall objective of this PhD project was to evaluate the functional properties of different plant foods (fruits, vegetables and grains) and of their bioactive compounds, trying to highlight the differences existing among plant foods of similar type.

Foods are mostly complex mixtures of macro and micro components organized in a structure that can trap active compounds, modulating their release or inhibiting their activity (8). Since it is known that the nutritional value of a food depends not only on the concentration of bioactive molecules, but also by their bioaccessibility, i.e. the fraction released from the food matrix during the digestive process, all studies described in this PhD thesis have the common characteristic to have evaluated not only the foods themselves, but mainly the products of their *in vitro digestion.*

In vitro digestion was performed using a standardized model that simulates the oral, gastric and duodenal phases of the digestive process, followed by the separation of the fraction containing the potentially bioavailable compounds. Although it is difficult to exactly mimic the physiological conditions taking place *in vivo*, this models allows to predict the bioavailability of different food components and is considered an easy, economic and reproducibly tool (9-10).

To study the products of *in vitro* digestion of foods represent a step ahead in the evaluation of their nutritional value for different reasons. First, phytochemicals exert their function synergistically, therefore the study of single molecules does not reflect what happens *in vivo* after food intake. For this reason the object of the research were not the single compounds, but foods in which they are contained.

Second, food digestibility depends also on the technological processing that food underwent before consumption. Submitting differently processed food to in vitro digestion allows the evaluation and comparison of the impact of processing on the nutritional value.

Third, foods are classified in general categories (i.e. apples, pears, etc) without considering that different varieties may have different composition and/or active components may have a different bioavailability,

In this PhD thesis, three types of plant food have been considering:

- mandarin juices obtained using different technological treatments;
- ancient and modern varieties of grains, that were used to make bread;
- peppers of different varieties.

In two studies, the variability of bioactive molecules and their effectiveness were investigated with a combined approach, including also the evaluation of their effectiveness in a biological system. The human hepatoma HepG2 cell line, widely used in biochemical and nutritional studies, was chosen as model system given that the liver is the organ mainly involved in xenobiotic metabolism (11).

In the first study, the impact of different technological processing on the antioxidant effect of mandarin juice was evaluated. Samples were *in vitro* digested and the mix of the bioavailable components was used for cell supplementation in basal condition and after exposure to an exogenous oxidative stress. The second study was focused on the investigation of the protective role of eight breads made with different whole ancient and modern grain flours. Breads were characterized and in *vitro* digested, then the digesta was used to supplement HepG2. The biological antioxidant and anti-inflammatory effects were evaluated in basal conditions and after a 2 h cell exposure to a mix of inflammatory agents. At last, the third study aimed to compare the digestibility and bioaccessibility of the main functional components of two different cultivars of sweet peppers.

Overall, the present PhD project allowed to consider some aspects that are often not considered or underestimated while evaluating the nutritional value of food: 1. The food matrix effect, including its intrinsic variability; 2. The bioavailability of components, and the impact of food processing; 3. The synergism among the different bioactive molecules; the biological response of cells.

Despite *in vitro* digestion and the use of cultured cells resemble only partially the *in vivo* situation, they are faster, less expensive, more ethical, and allow to select the most promising food and technologies before validation in clinical studies This research sets a new effective approach in the study of the nutritional properties of food.

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Impact of different processing on the nutritional value of mandarin juice

ABSTRACT

In recent years, consumer's and food industry interest in food having a high nutritional value is greatly increased. Notwithstanding, the impact of processing on food nutritional value is often underestimated.

Fruit juices are a good source of micronutrients and phytochemicals, that should be preserved during processing. Therefore, it is important to develop innovative technological treatments able to maintain the nutritional value of fruit juices. Homogenization is a process often used to improve physicochemical and functional properties of fruit juices.

The aim of this study was to investigate the impact of homogenization in different technological condition on the antioxidant properties of mandarin juice. The evaluation was not only performed on juices, but also on the product of their *in vitro* digestion. In addition, digested mandarin juices were supplemented to liver cultured cells, to evaluate the protective effect in a biological system.

Overall, data herein presented indicate that homogenization reduces antioxidant phenolics accessibility after *in vitro* digestion, but differences due to processing almost disappear when the antioxidant effectiveness of juices is evaluated in cultured cells.

Although further investigations are needed, our results highlight the importance of technological processing, and underline the needs of its evaluation to formulate food with a high nutritional value.

INTRODUCTION

Epidemiological studies suggest that diets rich in fruits and vegetables are related to a lower incidence of several chronic diseases.

Fruit juices are very popular in many countries and could represent an important strategy to improve the human diet as they retain most of the nutritional characteristics of the fruits from which they are extracted (1-2).

Mandarin juices are predominantly composed of water, have a low energy density and contain a range of key nutrients such as vitamin C, folate and phytochemicals. The major phytochemicals are phenolic compounds, a large group of secondary plant products with an aromatic ring bearing one or more hydroxyl substituents. The most common phytochemicals in mandarin juices are phenolic acids, flavanones (hesperidin, narirutin and didymin, usually present as glycosides form) and carotenoids (cryptoxanthin) (3-5). Several health-related properties have been ascribed to flavonoids, including antihyperglycemic (6), antimicrobial (7) and anticarcinogenic (8-10) activities, and they have been reported to protect against cardiovascular diseases (11-12). Moreover, *in vitro* and *in vivo* studies (13-15) showed that mandarin juices exert antioxidant effects.

The nutritional value of fruit juices mainly depends on the original fruit, but also the further processing has an important role. Therefore, there is increased interest towards the goal of obtaining foods with added nutritional value by new technologically strategies. The study of the relationship between food matrix and processing can help the development of new products detecting strengths and weaknesses of the system.

The objective of this work was to characterize mandarin juices obtained by different technological processing, and to evaluate their nutritional value by measuring their antioxidant capacity on human hepatoma cells (HepG2 cells). Oxidative stress contributes to the initiation and development of chronic diseases including cardiovascular disease, diabetes, dyslipidemia, and cancer (16-17). Therefore, bioactive compounds with antioxidant activity may influence numerous health outcomes by shifting the redox balance to reduce oxidative stress, (18).

Cultured cells are often used to evaluate the biological effects of food in pre-clinical studies. Particularly, HepG2 cells are considered a good experimental model since the liver has a central role in the metabolism of nutrients, xenobiotics and cytotoxic agents (19). In *in vitro* studies, cells are usually supplemented with discrete food-derived molecules and/or extracts, thus not resembling the *in vivo* situation. In fact, the use of extracts does not allow to monitor and evaluate all modification elapsing in the food during digestion. Digestion is a physiological event that is mandatory for obtaining bioavailable molecules, i.e. molecules available for absorption and it represents the first step of the process, since it allows the component release from the food matrix.

In this light, the overall nutritional value of foods cannot be simply ascribed to their concentration of nutrients and bioactive components, but also their bioaccessibility, i.e. their possible release from the food matrix. Digestion, and consequently food components bioaccessibility, is influenced by both gastrointestinal conditions (20-21) and chemical characteristics of the food matrix. Technological processes can improve the bioaccessibility of bioactive compounds, mainly through changes in the cell wall structure and properties. Plant matrix disruption and cell cluster disintegration due to applied processing steps determine phytochemical liberation and bioaccessibility (22).

To understand the impact of technological processing on the bioaccessibility of antioxidant molecules in mandarin juice, different juices were *in vitro* digested. Total antioxidant capacity (TAC) and total phenolic content (TPC) of not-digested and digested samples were determined, then, the digesta containing bioaccesible components were used for cell supplementation.

In order to evaluate the possible protective effects of the different mandarin juices, in the second part of the study supplemention to cultured hepatic cells was performed in both basal condition and after an exogenous oxidative stress. The effects of supplementation were verified by measuring cell

viability, reactive oxygen species (ROS) and reduced glutathione (GSH) intracellular content, and thiobarbituric acid reactive substances (TBARS) level in the media.

MATERIALS AND METHODS

Chemicals: Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza (Milan, Italy). 1-propanol was supplied by Carlo Erba (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy) and were of the highest analytical grade.

Mandarin juices preparation

Mandarin juices were prepared as previously described in (23). Briefly, ortanique fruit, a hybrid of tangerine and sweet orange (*Citrus sinensis x Citrus reticulata*) was harvested in an orchard located in Turis (Spain) and sent to the Department of Agro-Food Sciences and Technologies, University of Bologna, Cesena (Italy). The fruits were immediately washed by immersing them in tap water, drained and squeezed in an industrial extractor with finger cups. Raw juice was homogenized with a Manton-Gaulin pilot homogenizer at 20 MPa pressure, centrifuged, and the low pulp juice pasteurized at 63° C for 15 s for microbial inactivation.

The pasteurized juice was then submitted to three different technological processes: 1. homogenization at 20 MPa (MJ20); 2. homogenization at 20 MPa in the presence of 10% trehalose (MJ20+Tr); 3. homogenization at 20 MPa in the presence of *Lactobacillus salivarius* CECT 4063 (MJ20+Ls). In the following experiments, pasteurized mandarin juice (MJ) not undergoing additional technological treatment was also considered.

Homogenization reduces the particle size of fruit juice. It is widely used in the production of citrus juice to improve some quality factors such as viscosity, color, shelf-life, stability of the pulp, and to increase flavanone bioavailability (23).

Threalose addition stabilizes the juice suspension through the interaction with cloud compounds, and exertsa protective effect on various technological processes (24).

The CECT 4063 strain of *L salivarius* was chosen for its demonstrated activity against Helicobacter pylori infection (25).

Total antioxidant capacity (TAC) and total phenolics content (TPC) of the juices were determined as described below.

***In vitro* digestion**

The four different juices were digested *in vitro* according to the standardized method of Minekus *et al*. (26) that simulates oral, gastric and duodenal phases.

Digestion was performed twice for each kind of juices in a shaking water bath at 37°C; the resulting final digested solutions were centrifuged at 50,000 g for 15 min. The supernatants were filtered with 0.2 µm membranes, and an aliquot was sequentially ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (EMD Millipore, MA, US) in order to obtain mixtures of compounds which size is small enough to be potentially absorbable through the intestinal mucosa (<3K, bio-accessible fraction). Solutions derived from the two different digestions of the same type of juice were mixed and frozen until experiments.

Total antioxidant capacity (TAC) and total phenolics content (TPC) of the <3K fraction of digested juices were determined as described below.

Total antioxidant capacity (TAC)

TAC was measured using the method of Re *et al.* (27), based on the capacity of antioxidant molecules in the sample to reduce the radical cation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+). The decolorization of ABTS•+ was measured as the quenching of the absorbance at 734 nm. Values obtained were compared to the concentration-response curve of the standard Trolox solution and expressed as µmol of Trolox equivalents (TE)/ml.

Total phenolic content (TPC)

The concentration of total phenols was determined using Folin-Ciocalteau's method (28), adapted to a 96-well plate assay according to Dicko *et al.* (29) with slight modifications. Briefly, 45 µL of water were first pipetted into each well. Then, 5 µL of sample and 25 µL of 50% in water Folin-Ciocalteau (v/v) were added. After 5 min shaking, 25 µL of 20% (w/v) Na_2CO_3 aqueous solution and 100 µL of water were added to the mixture. The absorbance was measured after 60 min at 750 nm with a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Results were expressed as mg gallic acid equivalent (GAE)/ml of juice.

HepG2 cells culture and supplementation

HepG2 human hepatoma cells were grown in DMEM with 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 µg/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 flask, and culture medium was changed every 48 h.

Cells were seeded in 12-well plates at the concentration of 8×10^5 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 100 U/mL penicillin, 100 µg/mL streptomycin and the < 3KDa digested mixtures at the concentration of 100 µL/mL. To avoid interference due to vehicle, control cells (Ctrl) received a corresponding amount of a solution obtained from a "blank" digestion, that is an *in vitro* digestion performed without food.

Before determining whether the digested samples possessed hepatoprotective activity, the cytotoxicity of juices were measured by increasing its concentration and cells were supplemented with the highest non-cytotoxic concentrationIn some experiments, 24 h after supplementation cells were washed twice with warm DPBS and exposed for 1 h to 4 mM H_2O_2 in Earle's balanced salt solution (EBSS) (116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.38 mM $CaCl_2$, 0.39 mM $MgSO_4$) to cause an oxidative stress. The onset of oxidative stress was verified by quantification of ROS production and TBARS level.

In these experiments, a control condition was run by the exposure of not supplemented cells to EBSS without H_2O_2 for 1 h.

Cell viability

Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (30). The test is based on the capacity of mitochondrial dehydrogenase in viable cells to convert MTT reagent to a soluble blue formazan dye.

Briefly, cells were washed twice with DPBS, and MTT reagent in RPMI-1640 medium without phenol red (final concentration 0.5 mg/mL) added to cells. After 1 h of incubation at 37 °C, medium was completely removed, 1-propanol added to dissolve formazan product, and absorbance was measured against a propanol blank at 560 nm using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland). Cell viability was expressed as percent of corresponding control cells.

Measurement of intracellular ROS concentration

Intracellular ROS concentration was monitored spectrofluorometrically according to Valli *et al.* (31). Briefly, 30 min before oxidative stress, DCFH-DA, dissolved in absolute ethanol, was added to cells to a final concentration of 0.02 mM. DCFH-DA penetrates the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the non-fluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF in the presence of ROS. At the end of the oxidative stress, cells were washed twice with cold DPBS, lysed with 1 mL of cold Nonidet P-40 (0.25% in

DPBS), incubated for 30 min on ice under shaking and centrifuged at 14,000g for 15 min. DCF fluorescence intensity was detected (λex=485 nm, λem=535 nm) using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland), normalized for protein content in the sample and expressed as percent value of corresponding control cells.

GSH Content

After the oxidative stress, cells were lysed with 500 µL of cold Nonidet P-40 (0.25% in DPBS), incubated for 30 min on ice under shaking, and centrifuged at 14,000g for 15 min. One hundred microliters of the supernatant were incubated with 50 µL of DPBS and 50 µL of reagent buffer (160 mM sodium phosphate, 4 mM EDTA, 4% SDS and 500 µM DTNB) for 30 min. GSH was measured spectrophotometrically by reading the absorbance of the newly formed 5-thio-2-nitrobenzoic acid at 415 nm. The obtained results were compared to the concentration-response curve of standard GSH solutions, normalized for protein content in the sample and expressed as GSH/mg protein.

Thiobarbituric acid reactive substances (TBARS) concentration

TBARS, the end-products of lipid peroxidation, were assayed in EBSS as reported. After 1 h of stress with hydrogen peroxide, EBSS was removed, centrifuged at 400g for 3 min, and used for the assay. One hundred microliters of EBSS buffer was added to a mixture containing 100 µL of TCA (30% in 0.25N HCl), 100 µL of TBA (0.75% in 0.25 N HCl), and 3 µL of BHT (1% in ethanol). The mixture was heated for 10 min in a boiling water bath, allowed to cool, and the TBA adducts were detected fluorometrically (λex = 535 nm, λem = 595 nm). TBARS level was normalized for mg of proteins in each well and expressed as percent value of corresponding control cells.

Protein content

Protein content was determined according to Bradford (33), using bovine serum albumin in water as standard.

Statistical analysis

Statistical analysis of TAC and TPC was performed by the one-way ANOVA using Tukey's test as the post test. All other data were analyzed for statistical significance by the one-way ANOVA, using Dunnett's post-hoc test. Data obtained in cell cultures are reported as means ± SD of at least six samples derived from three independent cell cultures. All the analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA)

RESULTS

TAC and TPC of fresh and digested mandarin juices

Before digestion, no differences in TAC were detected among fresh juices (Fig. 1A), while MJ showed the highest TPC (Fig. 2A).

After digestion, TAC was significantly higher in digesta than in the corresponding fresh juices , with significant differences among juices (Fig 1B). TPC increased in MJ and MJ20+Tr digesta, and appeared higher than in the other juices (Fig 2B).

In digested samples, a significant correlation was observed between the TAC and TPC (Pearson correlation coefficient: r^2=0.9; p<0.05).

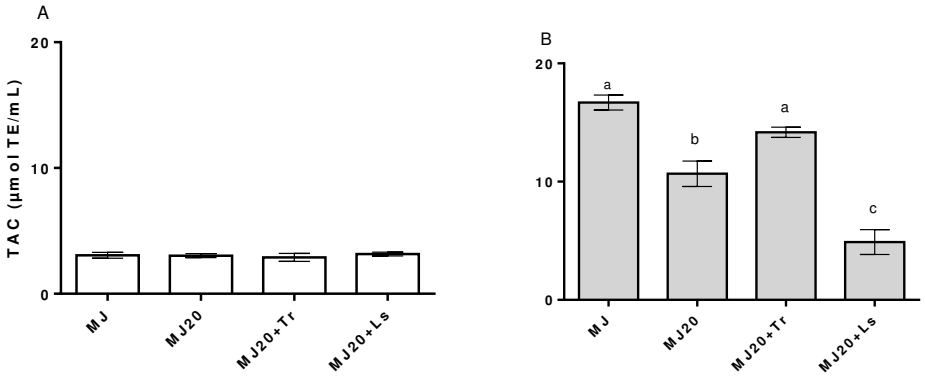

Figure 1. ***TAC of mandarin juices (A) and <3K digested mandarin juices (B).***
TAC is expressed as µmol of Trolox Equivalents (TE)/mL of juice. Data are means ± SD. Statistical analysis was by one-way ANOVA (A:ns, B: p<0.001) with Tukey's post-hoc test. Different letters indicate significant differences (at least p<0.05).

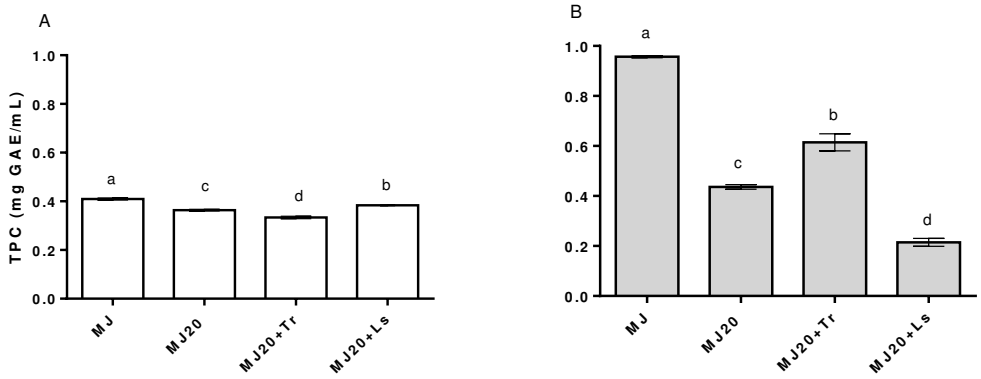

Figure 2. ***TPC of mandarin juices (A) and <3K digested mandarin juices (B).***
TPC is expressed as mg of Gallic Acid Equivalents (GAE)/mL of juice. Data are means ± SD. Statistical analysis was by one-way ANOVA (A and B: p<0.001) with Tukey's post-hoc test. Different letters indicate significant differences (at least p<0.05).

Effects of digested mandarin juices in cells

In basal condition, cell viability measured was not modified by the different supplementations (Fig. 3A). Compared to control cells in basal condition, incubation with 4 mM H_2O_2 resulted in a

significant decrease in cell viability in unsupplemented cells (USx), while no modification was observed in cells supplemented with the different juices (Fig. 3B).

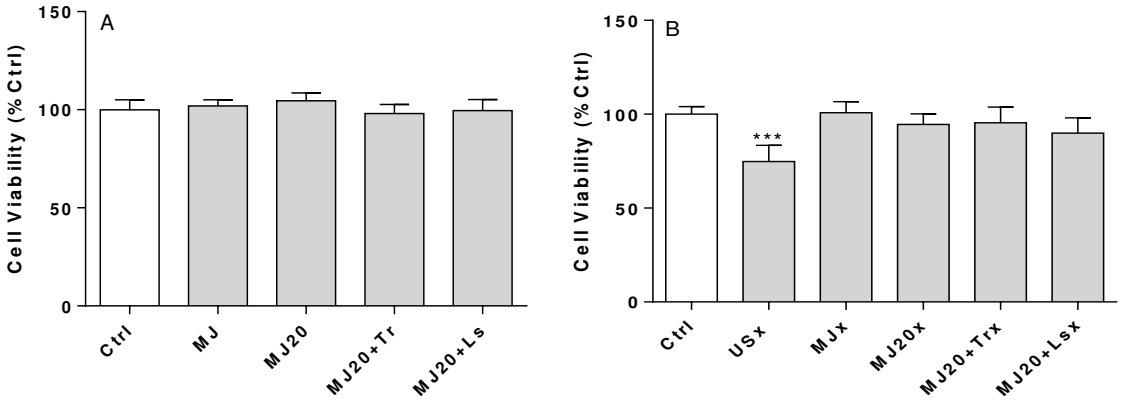

Figure 3. Cell Viability in basal (A) and stressed (B) conditions.
*Results are means ± SD (n = 6) and are expressed as percent of value in Ctrl cells (assigned as 100%). Statistical analysis was by one-way ANOVA (A: ns; B: p<0.001) with Dunnett's post-hoc test vs Ctrl (***p<0.001).*

In basal condition, all supplementations induced a significant reduction of ROS production compared to controls (Fig. 4A). Compared to basal control cells, treatment with H_2O_2 caused a significant increase of ROS concentration in all tested conditions (Fig. 4B). Comparing stressed cells, ROS concentration appeared significantly lower in MJ20 ($p<0.05$) and in MJ20+Ls ($p<0.01$) supplemented cells than in unsupplemented ones.

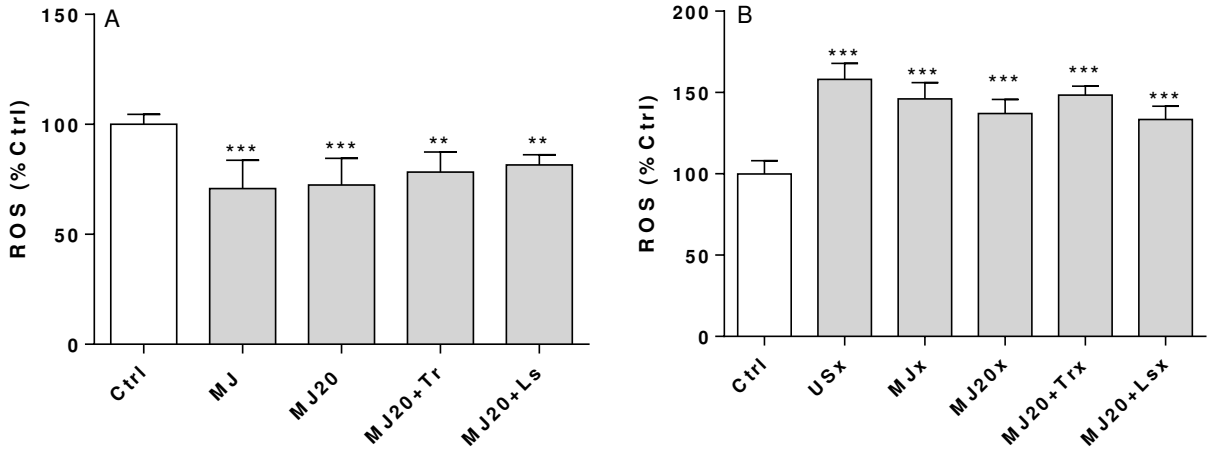

Figure 4: ROS intracellular concentration in basal (A) and stressed (B) conditions.
*Values are means ± SD (n = 6). Results were normalized for protein content, and are expressed as percent of value in basal control cells (assigned as 100%). Statistical analysis was by one-way ANOVA (p<0.001) with Dunnett's post-hoc test: ***p<0.001 and **p<0,01 vs Ctrl cells.*

In basal condition, no differences in GSH content were detected between control and supplemented cells, regardless the type of supplementation (Fig. 5A).

Upon H_2O_2 treatment, GSH level significantly decreased in cells supplemented with MJ and with the processed MJ20 and MJ20+Ls. (Figure 5B).

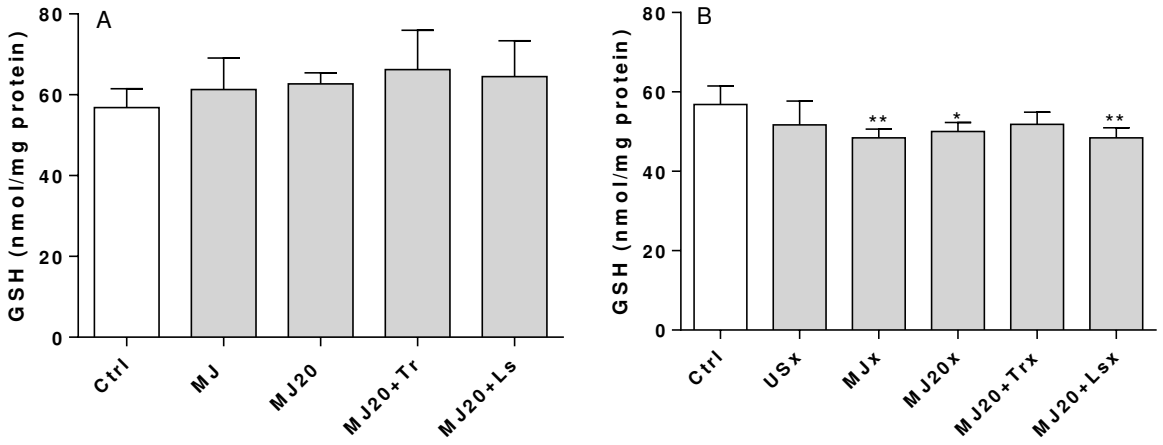

Figure 5: GSH in basal (A) and stressed (B) conditions.
*Results are means ± SD (n = 6). GSH concentration is expressed as nmol/mg protein and normalized for protein content. Statistical analysis was by one-way ANOVA (p<0.001) with Dunnett's post-hoc test (*p<0.05 and **p<0.01) vs corresponding Ctrl cells.*

In basal condition, all supplemented cells except MJ showed a significant reduction of TBARS level in the medium compared to controls (Figure 6A). The oxidative stress increased TBARS level in all cells, regardless supplementation (Fig. 6B).

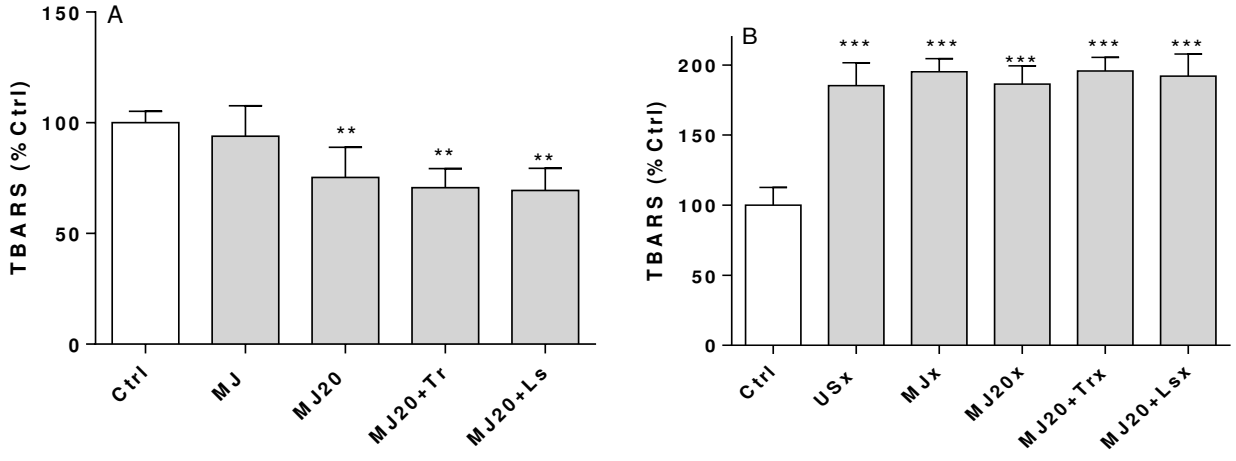

Figure 6: TBARS level in the media in basal (A) and stressed (B) conditions.
*Values are means ± SD (n = 6). Results were normalized for protein content, and are expressed as percent of TBARS concentration in the corresponding control cell (assigned as 100%) . Statistical analysis was by the one-way ANOVA (p<0.001) with Dunnett's post-hoc test (**p<0.01; ***p<0.001) vs corresponding Ctrl cells.*

DISCUSSION

In recent years, consumption of fruit juices is increasing, mainly due to their convenience. Since fruit juices have good concentration of nutrients (such as minerals and vitamins) and bioactive compounds (mainly carotenoids and phenolics), in many countries the Dietary Guidelines indicate them as a possible substitute of one out of the five recommended daily portions of fruit and vegetable. This indication is not included in the Italian Dietary Guidelines, due to concerns related to the low fiber content and the relatively high fructose concentration of fruit juices.

Among bioactive substances in fruit juices, phenolic compounds may have a major contribution to the health benefits of fruit juices consumption.

To exert biological effects, bioactive compounds must be released from the food matrix during digestion, so becoming bioaccessible. Technological processes could modify bioaccessibility, so it is important to identify suitable technological treatment, able to preserve both safety and nutritional value of fruit juices.

In the present study, the impact of different technological processing on the protective effect of mandarin juice was investigated. Mandarin juice was chosen as model fruit juice, and its effect was evaluated on cultured liver cells after *in vitro* digestion. Although it is difficult to exactly mimic the physiological conditions taking place *in vivo* in the gastro-intestinal tract, the use of the *in vitro* model has several advantages due to its simplicity, ease of application, and low cost.

The comparison between fresh mandarin juices and their corresponding digested samples allowed detecting a significant TAC and TPC increase in all 3k samples. This could depend not only on the release of bioactives during digestion, but also on the presence of antioxidants in the digestive juices (34).

As already reported (35-37), our results evidenced that processing can affect the release of components from the matrix. Fresh mandarin juice showed a higher TAC and TPC than homogenized juices. Among the latters, the juice homogenized in the presence of trehalose had the highest TAC and TPC, probably due to the trehalose interaction with cloud compounds and consequent stabilization of the suspension (38-39).

In basal condition, supplementation with the different mandarin juices was not toxic to cells. In addition, all juices appeared protective when cells underwent an exogenous oxidative stress. Oxidative stress, defined as an imbalance between pro-oxidants and antioxidants in the cell environment, has a key role in the pathogenesis of several chronic disease. In particular, oxidative stress has been implicated in the induction and progression of hepatic diseases, since the liver is the main target organ of several cytotoxic agents that can cause ROS- and free radical-mediated apoptosis (40). Hydrogen peroxide induces an array of cellular dysfunctions, including generation

of hydroxyl radicals, peroxidation of membrane lipids, deletion of GSH and protein thiol, and DNA damage, eventually leading to cell death (41-44).

In basal condition, supplementation with all juices significantly decreased ROS concentration, and had no effect on GSH level, suggesting that technological processes do not affect their biological activity. On the contrary, a decreased TBARS level was observed only in cells supplemented with the processed juices, the fresh one having no effect on this marker of oxidative stress.

Treatment with H_2O_2 caused a significant increase of ROS and TBARS levels in all cells, regardless supplementation. We can hypothesize that the induced stress was too strong to be efficiently reversed by juice components. Notwithstanding, some differences were detected comparing the different juices. In fact, compared to unsupplemented cells MJ20 and MJ20+Ls supplemented ones showed a lower ROS concentration, and GSH content significantly decreased in all supplemented cells except the MJ20+Trx ones.

Overall, data herein presented indicates that homogenization reduces antioxidant phenolics accessibility after *in vitro* digestion, but differences due to processing almost disappear when the antioxidant effectiveness of juices is evaluated in a biological system. Mandarin juice supplementation can modify the cell response mainly in basal condition, but without differences related to the technological treatment.

It is worth noting that the *in vitro* digestion model used in this study did not include the simulation of colonic digestion, where phenolics can be further metabolized by the microbiota (46). Therefore, results obtained on HepG2 cells could reflect only in part the overall effect on mandarin juice supplementation.

Further investigations are needed before conclusions can be drawn. Anyway, our results highlight the importance of technological processing and underline the needs of its evaluation to formulate food with a high nutritional value.

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Evaluation of the anti-inflammatory activity of ancient grains

ABSTRACT

Nowadays, the higher nutritional value of whole grains than refined grains is recognized, and epidemiological studies have clearly shown that consumption of whole grains and whole-grain-based products is associated with a reduction of the risk of developing many diseases.

Although in the last decade there is a renewed interest in the ancient varieties for producing high value food products with enhanced health benefits, the nutritional dominance of ancient vs modern grains is still controversial.

In this study, the anti-oxidant and anti-inflammatory effects of four different ancient grains and four different modern grains have been compared. To minimize differences due to agronomic and environmental factors, all grains were cultivated in the same location and growing season. Whole grain flours were obtained from grains, and used to make breads. After characterization, breads were *in vitro* digested, and the ultra-filtered digesta were supplemented to cultured liver cells.

The biological effects of digested bread were evaluated by measuring cell viability, ROS intracellular content, nitric oxide production and interleukin-8 secretion both in basal conditions and after 2 h exposure to a mix of inflammatory agents.

Overall, results herein reported clearly indicate that, despite the impossibility to discriminate breads made with ancient and modern based on their compositional characteristics, the effects exerted by their supplementation to cultured cells are different. Although *in vivo* studies are needed before drawing conclusions, this study represents a step ahead for the evaluation of the putative positive effects of ancient grains and for the formulation of cereal-based products with added nutritional value.

INTRODUCTION

In numerous countries food products derived from cereal grains constitute a major part of the daily diet. Particularly, wheat provides more nutritional sustenance to humans than any other crop and thus arguably remains the most important crop for humans (1).

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 (2-3).

In the last decade, there is a renewed interest in the ancient varieties for producing high value food products with enhanced health benefits (4). These beneficial properties are ascribed to higher levels

of proteins, lipids (mostly unsaturated fatty acids), soluble fibers, minerals, vitamins and phytochemicals, such as phytosterols and phenolic compounds (5-8). They are chiefly concentrated in the outer layers of grains and exist as soluble free compounds, soluble conjugates esterified to sugars and other low molecular weight molecules, and as insoluble forms bound to cell wall components (9-10). The highest concentration of health-promoting compounds in the outer layers could explain the reduction of the risk of developing many diseases, such as cardiovascular disease, diabetes, metabolic syndrome, and certain cancers (11-12).

Nowadays, the higher nutritional value of whole grains than refined grains is recognized (13), 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 (14-16) or discrete compounds derived from ancient wheats (17). This represents a limitation, since it is conceivable that the highest nutritional value and potential health benefit of ancient grains are not related to single compounds, but to their overall nutritional composition (18). 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, compositional differences existing among ancient grains and varieties of the same ancient species (19-21), often exacerbated by agronomic and environmental factors (22-23), could make difficult to generalize results obtained in a specific study. Overall, a definitive comparison between ancient and modern grains is still lacking.

In this study, the anti-oxidant and anti-inflammatory effects of four different ancient grains and four different modern grains have been compared. To minimize differences due to agronomic and environmental factors, all grains were cultivated in the same location and growing season. Whole grain flours were obtained from grains, and used to make breads. After characterization, breads were *in vitro* digested, and the ultra-filtered digesta were supplemented to cultured HepG2 liver cells. In some experiments, cultured cells were submitted to an exogenous inflammatory stress. The effects of the supplementation were investigated by measuring cell viability, reactive oxygen species (ROS) intracellular content, nitric oxide (NO) production, interleukin-8 (IL-8) and interleukin-10 (IL-10) secretion.

MATERIALS AND METHODS

Materials

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. Ingredients for bread formulation were purchased at local markets.

Grains

Four ancient (Kamut ^{TM} khorasan - KA; Spelt - SP; Marquis - MA; Turkey red - TU) and four modern grains (Fortuna - FO; Redwin - RE; Choteau - CH; Judy - JU) were considered.

Kernels were separated from the husk and cleaned from residues using sieves with different pores diameter. To obtain flour, grains were then milled with a small milling system (Molino Davide 4V, Novital, Italy). After every grinding each part was carefully cleaned in order to avoid contaminations, and flours were packed under vacuum and stored at 4°C.

Bread preparation

All breads 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, 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 breads. Once ready, breads were let to cool down at room temperature, cut in pieces and stored at -20 °C until analysis.

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

Table 1: Bread recipe

Bread nutritional composition and color analysis

Moisture, total nitrogen, carbohydrates, lipids, fibers and ashes were evaluated according to Baldini *et al.* (24). Selenium concentration in the different flours was determined by inductively coupled plasma-atomic emission spectrometry (25).

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.* (26) 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.* (27), and expressed as µmol of Trolox equivalents (TE)/ g. TPC was determined using Folin-Ciocalteau's

method, adapted to a 96-well plate assay according to Dicko *et al.* (28). Results were expressed as mg gallic acid equivalent (GAE)/ g.

The total carotenoid content (TCC) was determined using the method described by Valli *et al.* (29) with some modifications. Briefly, 1 g of bread was mixed with 4 mL of hexane-acetone (50:50, v/v), let 20 min at 40 °C under shaking, 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 β-carotene standard. Results were expressed as micrograms of β-carotene equivalents (β-CE)/ g.

The CIE system color profile of the eight breads was measured by a reflectance colorimeter (CR-400, Minolta, Italy) using illuminant source C (30). 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 range from 0 black to 100 white), a* the redness (that range from green associated with negative values to red associated with positive values) and b* the yellowness (that range from blue associated with negative values to yellow associated with positive values). The colorimeter was calibrated using a standard white ceramic tile.

In vitro digestion

The breads were digested *in vitro* according to the standardized method of Minekus *et al.* (31). 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 ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (EMD Millipore, MA, US) (<3KDa, bio-accessible fraction). Ultrafiltered solutions derived from two different digestions of the same bread were mixed and frozen at -20°C until experiments. TAC and TPC of the digesta from different breads were determined as described above.

HepG2 cells culture and supplementation

HepG2 cells were grown in DMEM with 10 % (v/v) fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained in a humidified atmosphere (95 % air and 5 % CO_2) at 37°C. Once a week cells were split 1:20 into a new 75 cm^2 flask, and culture medium was changed every 48 h.

Cells were seeded in 12-well plates at the concentration of 8×10^5 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 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. Cells were also supplemented with 4mM sodium salicylate (SS) to compare the effect of digested breads to the effect of a well-known anti-inflammatory agent. To avoid interference due to vehicle, some cells received a corresponding amount of a solution obtained from a "blank" digestion, that is an *in vitro* digestion performed without food. Preliminary experiments were performed to check possible differences in term of cell viability and cytokines 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).

In some experiments, 24 h after supplementation media were removed and cells were incubated for two additional hours with new DMEM containing the inflammatory agents lipopolysaccharide (LPS, 100 ng/mL), interleukin-1β (IL-1β, 10 ng/mL), and tumor necrosis factor α (TNF-α, 10 ng/mL) (32).

After 2 hours media were removed, cells scraped-off and maintained at -20 °C until analyses.

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.* (33). Results were expressed as percentage of value obtained in Ctrl cells.

ROS intracellular concentration

Intracellular ROS concentration was monitored spectrofluorometrically as described in details by Valli *et al.* (34). Briefly, DCFH-DA (2mM) in absolute ethanol was kept in the dark at -20°C until use. In basal condition, 10 µL DCFH-DA/mL medium were added to HepG2 cells 30 min prior to digesta supplementation. In inflammatory 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 on supernatants (λex=485 nm, λem=535 nm) using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland), normalized for protein content in the sample, and expressed as percent of value in Ctrl cells.

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. Results were normalized for protein content in the well, and expressed as μM of nitrites and nitrates.

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 both basal condition and after cell treatment with the inflammatory agents by AlphaLISA kits (IL-10 and IL-8 Immunoassay Research Kits; Perkin Elmer Inc., Waltham, MA, USA) following the manufacturer's instructions (35). 96-microwell plates (96 1/2 AreaPlate from Perkin Elmer) were used and read using an EnSpire™ plate reader (Perkin Elmer Inc., Waltham, MA, USA). Results were normalized for protein content in the well, and expressed in pg/ mL medium/mg protein.

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 (36) using bovine serum albumin (BSA) as standard.

Statistical analysis

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

RESULTS

Bread nutritional composition

The nutritional composition of the different flours is presented in Table 2. MA, CH and FO breads had the highest content in total nitrogen, and MA and FO the lowest content of available carbohydrates. Water, lipids, ash, energy and selenium content was similar among the different breads.

	KA	MA	SP	TU	CH	FO	RE	JU
Water (g/100g)	27.96 ± 1.40 [a]	30.18 ± 1.51 [a]	26.83 ± 1.34 [a]	30.74 ± 1.54 [a]	28.92 ± 1.45 [a]	29.66 ± 1.48 [a]	29.19 ± 1.46 [a]	29.66 ± 1.48 [a]
Total Nitrogen (g/100g)	12.70 ± 0.64 [c,d]	15.39 ± 0.77 [a]	13.37 ± 0.67 [b,c]	12.56 ± 0.63 [c,d]	14.91 ± 0.75 [a,b]	15.30 ± 0.77 [a]	11.27 ± 0.56 [d]	12.21 ± 0.61 [d]
Carbohydrates (g/100g)	49.85 ± 1.66 [a]	42.74 ± 1.89 [c]	49.66 ± 1.66 [a,b]	47.06 ± 1.80 [a,b,c]	45.23 ± 1.81 [a,b,c]	44.76 ± 1.82 [b,c]	49.13 ± 1.73 [a,b]	47.87 ± 1.77 [a,b]
Lipids (g/100g)	1.67 ± 0.17 [a]	1.67 ±0.17 [a]	1.75 ± 0.18 [a]	1.61 ± 0.16 [a]	1.53 ± 0.15 [a]	1.52 ± 0.15 [a]	1,58 ± 0.16 [a]	1.62 ± 0.16 [a]
Fibers (g/100g)	5.90 ± 0.59 [b]	7.97 ±0.80 [a]	6.17 ± 0.62 [a,b]	6.09 ± 0.61 [a,b]	7.32 ± 0.73 [a,b]	6.62 ± 0.66 [a,b]	6.69 ± 0.67 [a,b]	6.36 ± 0.64 [a,b]
Ash (g/100g)	1.96 ± 0.29 [a]	2.05 ±0.31 [a]	2.22 ± 0.33 [a]	1.94 ± 0.29 [a]	2.09 ± 0.31 [a]	2.15 ± 0.32 [a]	2.13 ± 0.32 [a]	2.29 ± 0.34 [a]
Energy (Kcal/100g)	277 [a]	263 [a]	280 [a]	265 [a]	269 [a]	267 [a]	269 [a]	268 [a]
Selenium (mg/100g)	0.056 ± 0.024 [a]	0.034 ±0.014 [a]	0.079 ± 0.012 [a]	0.045 ± 0.019 [a]	0.034 ± 0.014 [a]	0.040 ± 0.017 [a]	0.054 ± 0.023 [a]	0.052 ± 0.022 [a]

Table 2. Nutritional composition and Selenium content of the different breads.
Data are means ± SD (n = 3). Statistical analysis was carried out by the one way ANOVA with Tukey's HSD post-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 $P < 0.05$).

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

	KA	MA	SP	TU	CH	FO	RE	JU
L*	49.84 ± 1.24 [d,e]	47.57 ± 0.25 [e,f]	57.56 ± 0.80 [a]	55.40 ± 1.29 [a,b]	46.50 ± 1.20 [f]	52.17 ± 2.74 [c,d]	53.96 ± 0.04 [b,c]	57.29 ± 0.04 [a]
a*	3.46 ± 0. 19 [f]	6.20 ± 0.04 [a]	4.79 ± 0.25 [d,e]	5.38 ± 0.17 [c]	5.76 ± 0.08 [b]	5.01 ± 0.08 [d]	5.37 ± 0.07 [c]	4.68 ± 0.06 [e]
b*	21.45 ± 0.10 [a]	17.28 ± 0.03 [d,e]	19.27 ± 0.76 [b]	17.99 ± 0.60 [c,d]	16.22 ± 0.42 [f]	17.03 ± 0.44 [e]	18.73 ± 0.05 [b,c]	19.17 ± 0.11 [b]

Table 3. Breads color profile.
Data are means ± SD (n=3). Statistical analysis was by one-way ANOVA($p< 0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

As shown in Figure 1, the TAC, TPC, and TCC were specie-specific, with no clear discrimination between ancient and modern grains. 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.

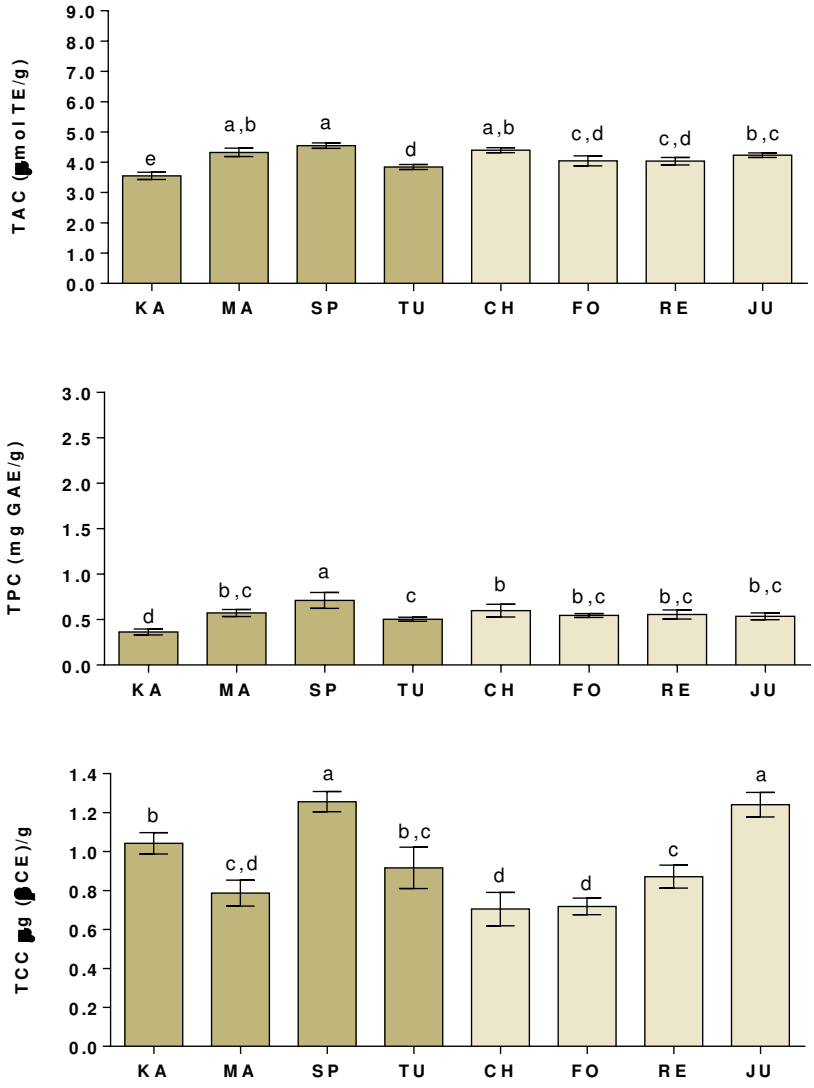

Figure 1. TAC, TPC, and TCC of breads.
Data are means ± SD (n=3). Statistical analysis was by one-way ANOVA ($p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

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 ancient and modern grain bread digesta, except in modern RE bread which showed significantly lower TAC (Figure 2). 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 detection limit, probably due to the low bioaccessibility of these molecules, as recently reported by Corte-Real *et al* (37).

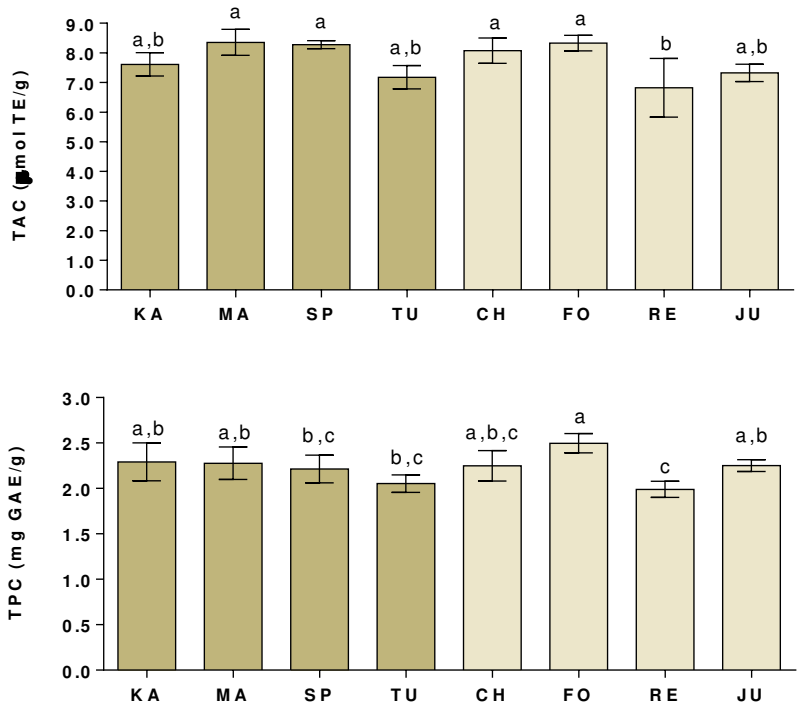

Figure 2. TAC and TPC of digested breads.
Data are means ± SD (n=3). Statistical analysis was by one-way ANOVA ($p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

Effects on cultured cells – basal condition

Cell viability of cells supplemented with bread made with two ancient grains (KA and SP) was significantly higher than in controls. On the contrary, SS caused a significant decrease in cell viability (Figure 3A).

Compared to controls, supplementation with all breads except MA and FO decreased ROS intracellular concentration (Figure 3B). NO secretion in the cell media increased in cells supplemented with KA and TU (ancient grains) and CH (modern grain), and mainly in SS supplemented cells (Figure 3C).

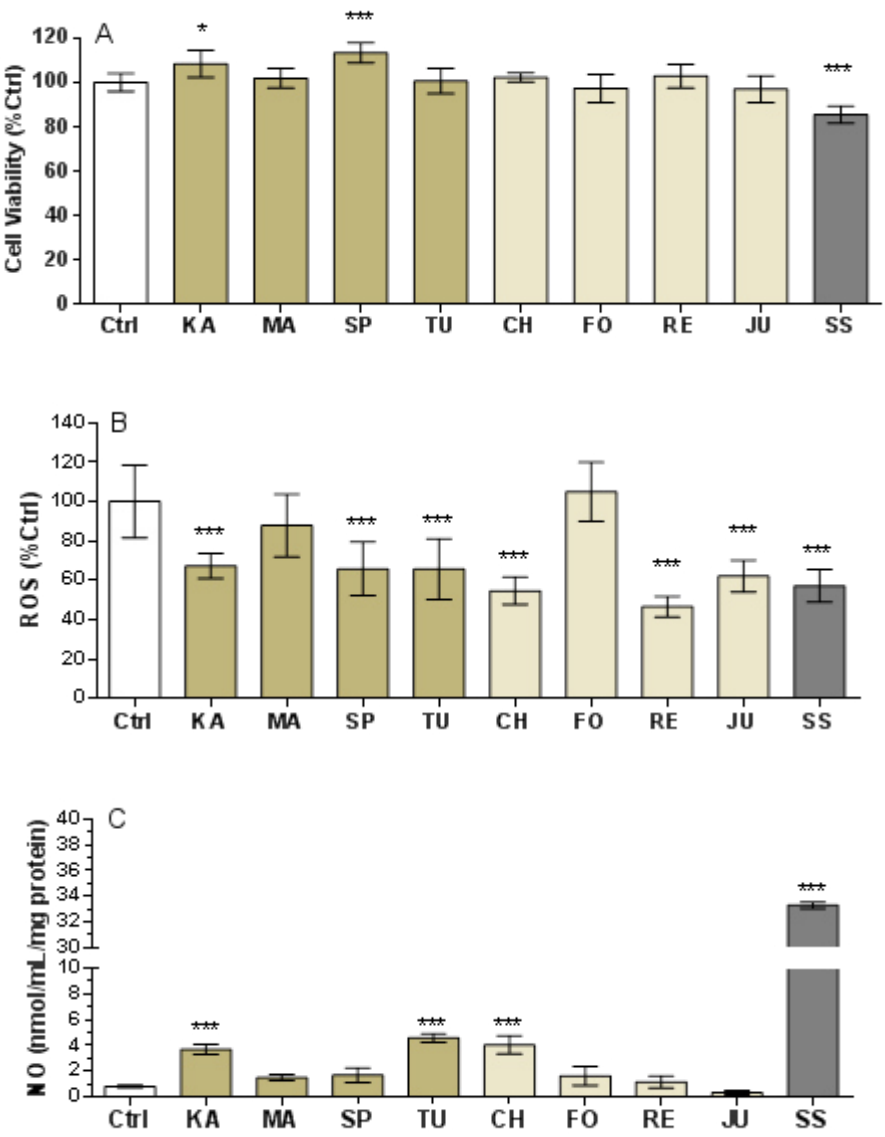

Figure 3: Effects on cells in basal condition.

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

Within the modern grain supplemented group, secretion of pro-inflammatory IL-8 was significantly higher in 3 out of 4 supplemented cells than in control cells, and in cells supplemented with SS. On the contrary, the IL-8 secretion was significantly lower in KA supplemented cells than in controls (Figure 4).

In all cells, IL-10 secretion was very low, below the detection limit (data not shown).

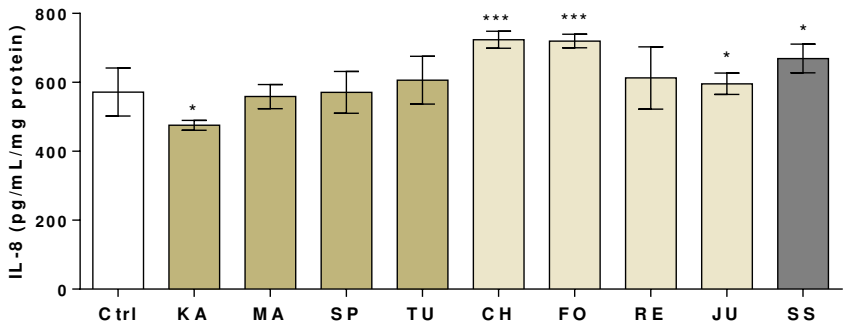

Figure 4: Interleukin-8 (IL-8) secretion in the cell media in basal condition.
*Data are means ± SD (n=6). Results are expressed as pg IL-8/ mL medium/ mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, and ***$p<0.001$ vs corresponding control cells.*

Effects on cultured cells – inflamed condition

In inflamed cells, no significant differences in cell viability were detected between control and digested bread supplemented cells, and the detrimental effect of SS was still present (Figure 5A). Compared to corresponding controls, ROS concentration was significantly increased in all cells supplemented with breads made with modern grains, except CH ones (Figure 5B).

NO production was not influenced by the different supplementation except JU and SS, which caused a significant increase of NO concentration in the media (Figure 5C)

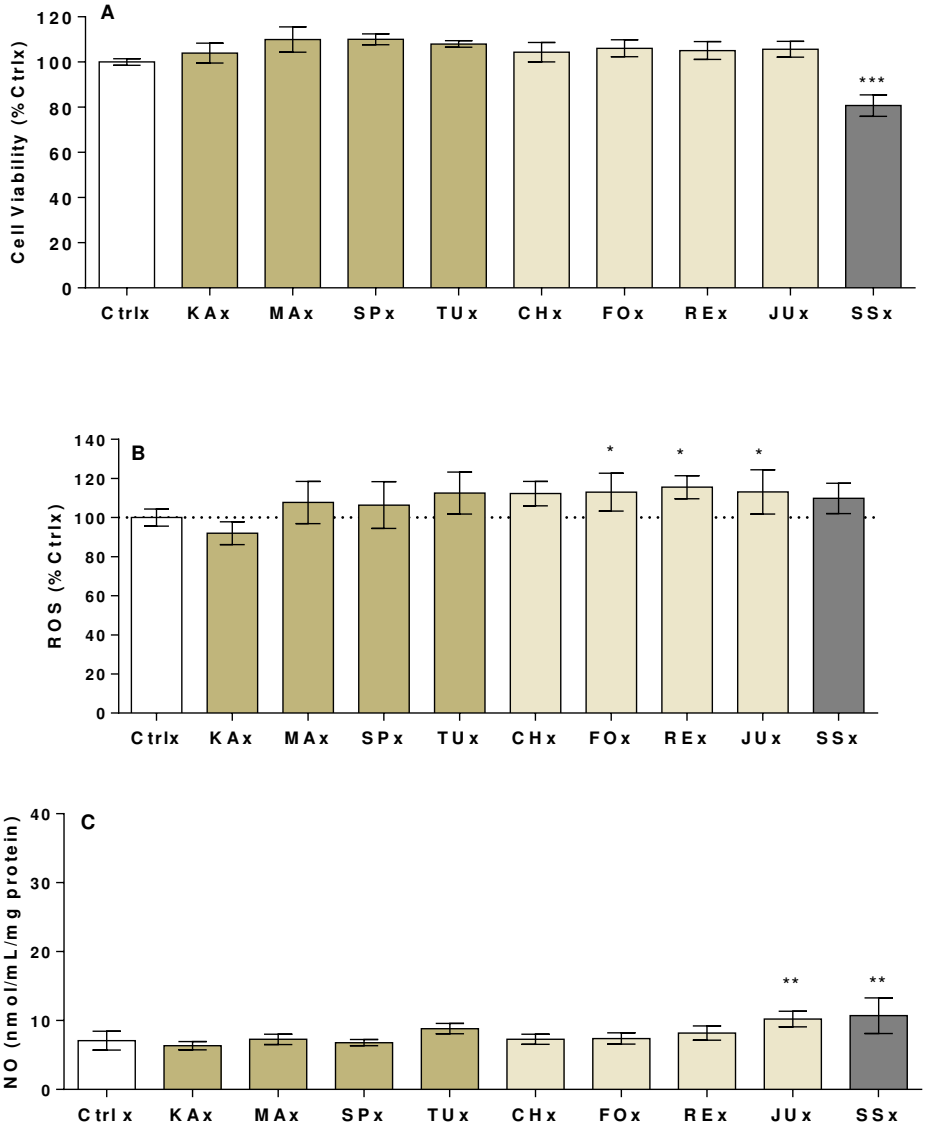

Figure 5: Effects on cells in inflammed condition.
*Results are means ± SD (n=6). Panel A: cell viability. Results are expressed as percent of value in the corresponding control cells (assigned as 100%). Panel B: ROS intracellular concentration. Results were normalized for protein content in the sample, and are expressed as percent of value in the corresponding control cells (assigned as 100%).Panel C: NO secretion. Results are expressed as nmol NO/ mL medium/ mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs corresponding control cells.*

The inflammatory stimulus greatly increased IL-8 production in all cells compared to their basal counterparts. Compared to the corresponding control cells, IL-8 production was significantly higher in SS and modern grain supplemented cells except CH ones, while no differences were detected among controls and cells supplemented with ancient grains (Figure 6).

Even in inflamed condition IL-10 secretion was below the detection limit (data not shown)

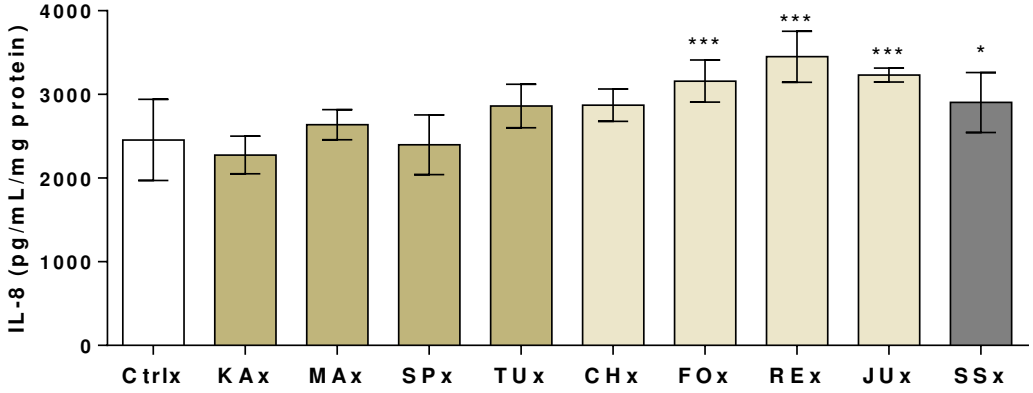

Figure 6: Interleukin-8 (IL-8) secretion in the cell media in inflamed conditions.
*Data are means ± SD (n=6). Results are expressed as pg IL-8/ mL medium/ mg protein in the well. Statistical analysis was by one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, and ***$p<0.001$ vs corresponding control cells.*

DISCUSSION

In order to point out differences among the different grains, breads made with the corresponding flours were characterized in term of nutritional composition, color profile, TAC, TPC and TCC.

Analyses evidenced a substantial similarity of nutritional profiles and selenium content among breads made with the different wheat varieties. Although these results are in disagreement with previous report (38), they could be 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 (39-40) underlining that the degree of yellowness in wheat grain and its end products is affected by carotenoids degradation during processing (41). In this study the highest L* and TCC were detected in SP and JU breads.

According to Dinelli *et al* (42), a high variability of antiradical activity and polyphenol content was observed among the investigated breads, and a significant correlation was detected between TAC and TPC, as reported by Adom and Lui (43). Anyway, it was not possible to discriminate ancient and modern grains based on their TAC, TPC and TCC values.

Digestion process and pH conditions result in starch hydrolysis, proteolysis and releasing phenolics from their conjugation forms as well as cell wall matrices (44-46). 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 ancient and modern grains based on these parameters.

Since several of the phytochemicals in whole grain have been reported to exert antioxidant and anti-inflammatory effects (47), in the second part of the study digested breads were supplemented to cultured liver cells to evaluate their protective role in basal condition and after an inflammatory stress.

In basal condition, cell viability increased in cells supplemented with Kamut ™ Khorasan bread and spelt bread, while no effect of bread supplementation was observed in inflamed cells.. Supplementation with SS caused a significant decrease in cell viability in both conditions. A similar effect of SS in HepG2 cells has been already reported by Raza *et al* (48), due to alteration in mitochondrial respiratory function, cell cycle arrest and increasing oxidative stress.

In basal condition, the supplementation with the most of breads and with SS decreased ROS production, so suggesting a protective effect. On the contrary, this putative protective effect disappeared after the inflammatory treatment, and ROS concentration was similar in control and in cells supplemented with bread made with ancient grains. On the contrary, ROS concentration was higher in cells supplemented with 3 out of 4 breads made with modern grains (FO, RE and JU).

It has been reported that SS increases NO production in HepG2 cells, via the modulation of inducible nitric oxide synthase (iNOS) (49). In agreement, in this study cells supplemented with SS showed an increased NO production in both basal and inflammatory condition. Phenolic compounds also modulate iNOS (50-51). This could explain the observed increase in NO production in cells supplemented with the ancient KA and TU and the modern CH compared to controls in basal condition. In inflamed condition, NO production was similar in control and supplemented cells, except the JU supplemented ones. The Janus role of NO is well known, and it can be protective or harmful depending on the kind of insult and the amount of NO (52). In this light, it is interesting to note that in all cells except the SS treated ones, NO concentration was higher after 2 h exposure to the inflammatory stimulus than after 24 h supplementation in basal condition. On the contrary, after 24 h treatment with SS NO concentration was about 35 nmol/mL medium/mg protein, and it decreased to about 10 nmol/mL medium/mg protein after 2 h inflammation.

Since HepG2 cells have been reported to produce IL-8 and IL-10 in response to specific stimulation (29), these two cytokines were chosen as markers to further evaluate the possible modulation of inflammation by the different breads. 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 (53), whereas IL-10 is a

prototypical regulatory cytokine exerting several immune-modulatory effects, and cereals have been shown to stimulate its production in monocytes (54).

The production of many pro-inflammatory cytokines, including IL-8, is mainly under control of the transcription factor NF-kB (55), which plays a critical role in the expression of many genes involved in immune and inflammatory responses activation in HepG2 cells. Callejas *et al* (49) evidenced that NSAIDs, including SS and aspirin, fail to interfere with NF-kB. This could explain the increase of IL-8 secretion observed in SS supplemented cells in both basal and inflammatory conditions.

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. The inflammatory stimulus greatly increased IL-8 secretion in all cell groups compared to the basal counterparts. Compared to inflamed control cells, IL-8 concentration was higher in the media of cells supplemented with bread made with modern grains, except CH.

IL-10 secretion was below the detection limit in all tested conditions.

Overall, results herein reported clearly indicate that, despite the impossibility to discriminate breads made with ancient and modern based on their compositional characteristics, the effects exerted by their supplementation to cultured cells were different.

Different markers were used to identify the protective role of bread (cell viability, ROS concentration, NO secretion and IL-8 production), and two ancient grains (KA and SP) ameliorated the most of them in basal condition. In inflamed condition, no differences were detected between controls and cells supplemented with ancient grains, while the most of modern grains had a detrimental effect on ROS concentration and IL-8 production.

The observed protective effects of KA is in agreement with a previous studies in cultured cells (29), animals (56), and humans (57) and with a recent review (4) reported in Appendix.

In addition to natural compounds, the protective activity in ancient grain-based foods could be due to increased browning reaction during baking and toasting processes (3). This effect 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 (through the activation of the gene expression of superoxide dismutase) and anti-inflammatory factors (58-59). On the other hand, advanced glycation end products (AGEs) are pro-inflammatory and toxic (60). As reported in a previous study (29) it is conceivable that the use of different flours led to a different production of both MRPs and AGEs, contributing to the different antioxidant and anti-inflammatory effect.

Although this study does not allow to evidence exactly which flour components are the protective ones, this must not be considered as a limitation since it is known the possible synergism among the different molecules and the importance of some aspects related to the food matrix.

Overall, our results confirm the potential health effects of ancient grains, particularly KA and SP. Although the use of *in vitro* digestion reduced in part the distance from the physiological situation *in vivo*, further investigations are needed. 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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In vitro digestibility and bioaccessibility of bioactive compounds in different cultivar of Sweet Pepper (*Capsicum annuum*)

ABSTRACT

The high content of antioxidant bioactive compounds such as vitamin C, carotenoids, phenolics and flavonoids has increased the interest of consumers, food industry and the scientific community in sweet pepper fruits (*Capsicum annum L.*). However, a huge number of *Caspicum annuum* cultivars is grown worldwide, and it is known that the concentration of the bioactive compounds can be different among varieties of the same vegetable and may be affected by the harvesting period. In addition, the possible health benefits of phytochemicals depend not only on their concentration in the food, but also on their availability in the target tissue and therefore on their digestibility, that is the amount of the food constituent that is digested and released from the solid food matrix in the gastrointestinal tract.

The current study was designed to evaluate and compare digestibility and bioaccessibility of the main functional components of two different cultivars of sweet pepper (Lamuyo and Cornelio) cultivated in Italy. Peppers were submitted to an *in vitro* digestion using the standardized model that simulates oral, gastric and duodenal digestion. Digested samples were centrifuged and filtered on 0.2 µm membranes (TQ digested samples) and an aliquot was sequentially ultrafiltered (< 3K fractions), allowing the separation of potentially bioavailable compounds. Total phenolic content (TPC), vitamin C concentration, total carotenoid content and antioxidant activity (TAC) were assessed in fresh peppers, TQ digested samples and < 3K digested fractions, then digestibility and bioaccessibility indices of bioactive components were calculated.

Results showed that TPC and TCC in fresh peppers were different between the two cultivars.

TAC increased in digested samples compared to the not digested counterparts, suggesting the release of antioxidant components from the food matrix. Furthermore, red Lamuyo showed higher digestibility and bioaccessibility indices of TPC while Cornelio higher digestibility index of Vitamin C than Lamuyo peppers.

At present, food are nutritionally evaluated on the basis of their chemical characteristics, without taking into account that their components must be released during digestion, and the food matrix can have a great impact on the entity of the release, and therefore on bioaccessibility. Considering the digestive process this work sets a new effective approach in the study of the nutritional properties of food.

INTRODUCTION

Peppers (*Capsicum annuum*) are one of the most widely consumed vegetables for their combination of flavor and nutritional value (1). They are a good source of vitamin C, vitamin E, folate (2) and carotenoids that include β-carotene (pro-vitamin A) and oxygenated carotenoids such as zeaxanthin, capsantine, capsorubin and cryptocapsin. Carotenoids are lipid soluble compounds derived from the isoprenoid pathway and share a carbon isoprene backbone with a variety of ring structures, which in the case of pepper fruits are stored in the chromoplasts. Even though capsanthin seems to be the most abundant carotenoid in peppers, other studies reported different carotenoid composition. These findings could be explained by variations in sample preparation (whole fruit including seeds or not), extraction and quantification methods, the use of fresh or dehydrated fruits (3-4).

In addition, peppers were reported to contain high quantities of neutral phenolic compounds or flavonoids (quercetin, luteolin, and capsaicinoids) which are important for a variety of plant defense responses (5). They have been recognised as being beneficial for human health, due to their antioxidant properties, which protect against the oxidative damage to cells and thus prevent the development of common degenerative diseases such as cancer, cardiovascular diseases, cataracts, diabetes and Alzheimer's (6-8).

Currently, a broad number of varieties of peppers are available worldwide, most of which changes for shape and color. Levels of bioactive compounds can be affected by maturity stage, growing conditions, genotype as well as storage and processing. The phytochemical changes that occur during maturation and the resultant effect on antioxidant activity are important considerations that may affect pepper's nutritional value (9-10).

Moreover, it is noteworthy that the possible health benefits of phytochemicals depend not only on their concentration in food, but also on their digestibility, that is the amount of the food constituent that is released from the food matrix in the gastrointestinal tract. During digestion, many functional components are altered and transformed into other compounds. Numerous mechanisms may be responsible for the digestibility of food components, including physicochemical factors as the release from plant tissues and the solubility in gastrointestinal fluids (11-14).

The objective of the present work was to assess and compare the digestibility of two varieties of peppers, namely Lamuyo and Corno di Toro.

Digestibility was assessed by measuring the levels of phenolic compounds (TPC), Vitamin C, and carotenoids (TCC) in the raw peppers and in the corresponding digested samples. To do it, peepers were in vitro digested in order to reproduce the modifications of the food matrix occurring *in vivo* in the gastrointestinal tract. The total antioxidant capacity (TAC) of raw peppers and digested samples was also measured. Digestibility and bioaccessibility indices were then calculated and compared.

MATERIALS AND METHODS

Chemicals

All chemicals, reagents, and solvents were purchased from Sigma-Aldrich (Milan, Italy)

Sample preparation

Two pepper varieties cultivated in Italy were considered: Corno di Toro (CORN) and Lamuyo (LAM). In both varieties, red and yellow peppers were analyzed (CORN R and CORN G, LAM R and LAM G).

For both varieties and colors, two harvesting periods were considered, February and October.

Three fresh whole peppers, chosen randomly from each type to consider intrinsic variability, were cut and chopped into small pieces. Twenty–five grams were weighted and submitted to *in vitro.* One g of the same fresh sample was extracted according to Danesi *et al.* (15) with a final volume of 12 mL ethanol/water (70:30) acidified with 0.1 % HCl.

In vitro digestion

Peppers were submitted to an *in vitro* digestion using the standardized model of Minekus *et al.* (16) that simulates oral, gastric and duodenal phases, followed by separation of supernatant (Figure 1). Digestive juices were SSF (Simulated Salivary Fluid, ph 7), SGF (Simulated Gastric Fluid, ph 3) and SIF (Simulated Intestinal Fluid, ph 7). Digestion was performed in a shaking water bath at 37°C; the resulting final digested solutions were centrifuged at 50,000 g for 15 min. The supernatants were filtered with 0.2 µm membranes (TQ fraction) and an aliquot was sequentially ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (EMD Millipore, MA, US) in order to obtain mixtures of compounds which size is small enough to be potentially absorbable through the intestinal mucosa (<3K, bio-accessible fraction). Filtered and ultrafiltered digesta were frozen at -20°C until experiments.

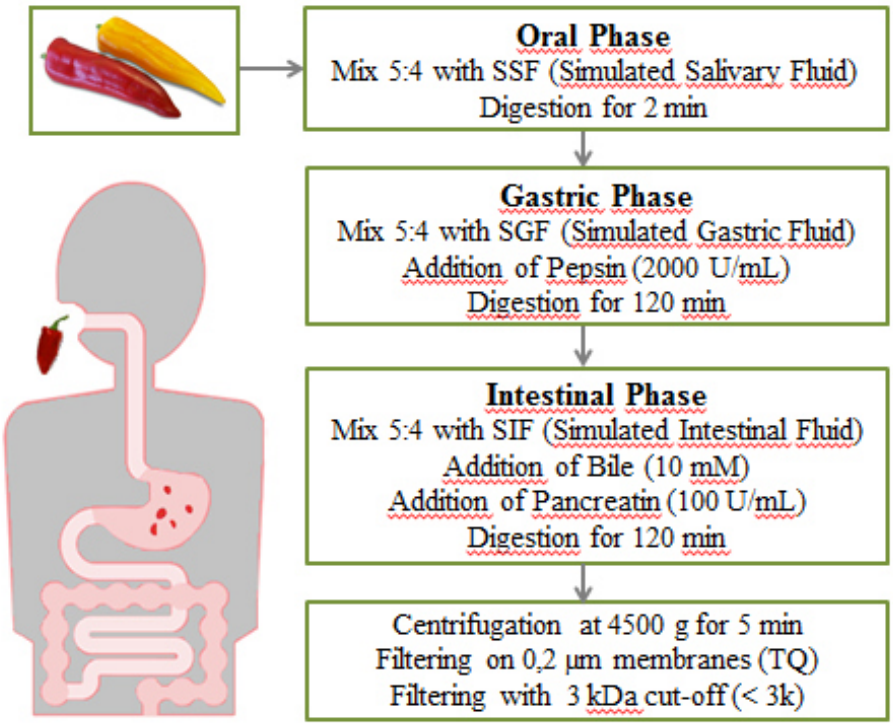

Figure 1. Flowchart of the simulated human in vitro digestion method

Total antioxidant capacity (TAC)

TAC was measured in fresh and digested samples using the method of Re *et al.* (17), based on the capacity of antioxidant molecules in the sample to reduce the radical cation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+). The decolorization of ABTS•+ was measured as the quenching of the absorbance at 734 nm. Values obtained were compared to the concentration-response curve of the standard Trolox solution and expressed as µmol of Trolox equivalents (TE)/g.

Total phenolic content (TPC)

The concentration of total phenols was determined in fresh and digested samples using the Folin-Ciocalteau's method (18), adapted to a 96-well plate assay according to Dicko *et al.* (19) with slight modifications. Briefly, 45 µL of water were first pipetted into each well. Then, 5 µL of sample and 25 µL of 50% in water Folin- Ciocalteau (v/v) were added. After 5 min shaking, 25 µL of 20% (w/v) Na2CO3 aqueous solution and 100 µL of water were added to the mixture. The absorbance was measured after 60 min at 750 nm with a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Results were expressed as mg gallic acid equivalent (GAE)/g.

Total carotenoid content (TCC)

The total carotenoid content (TCC) was determined in fresh and digested samples using the method described by Valli *et al.* (20) with some modifications. Briefly, 1 g of pepper was mixed with 40 mL of hexane-acetone (50:50, v/v), let 20 min at 40 °C under shaking, vortexed at high speed, sonicated, vortexed again, and centrifuged at 120 × *g* for 3 min. For the evaluation of TCC in digested peppers, 2 ml of sample was mixed with 5 ml of hexane-acetone and with 3 mL of NaCl 10%. 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)/g.

Vitamin C concentration

The Vitamin C concentration was measured in fresh and digested samples by iodine titration (21). Samples were titrated while stirring with iodine solution using starch indicator for the detection of the end point. As the iodine is added during the titration, the ascorbic acid is oxidized to dehydroascorbic acid, while the iodine is reduced to iodide ions. Titration was carried out till the formation of persistent blueblack starch-iodine complex, and vitamin C concentration was expressed as mol/g fresh sample.

Digestibility and bioaccessibility indices

The digestibility index was calculated based on total phenolic content, vitamin C concentration and TCC in the fresh samples and corresponding TQ digested fraction using the following equation:

(concentration in TQ digested fraction/ concentration in fresh sample) x 100.

This index indicates the efficiency of the digestive process in releasing the considered compound(s) from the food matrix.

The bioaccessibility index was calculated based on total phenolic content and vitamin C concentration in the fresh samples and corresponding <3K digested fraction using the following equation:

(concentration in <3K digested fraction/concentration in fresh sample) x 100.

This index estimates the percentage of potentially bioavailable compound(s), i.e. the fraction released from the matrix and hydrolyzed having a size that is small enough to be potentially absorbable through the intestinal mucosa (22).

Statistical analysis

All data are reported as means ± SD of biological and technical replicates for peppers harvest in February (n = 4), and of biological replicates for peppers harvested in October (n=5). Data were analyzed for statistical significance by the Student's t test.

RESULTS

Total Phenolic Content (TPC)

Table 1 reports TPC of the different fresh peppers from the two harvesting periods. In the first study, TPC appeared significantly higher in CORN R and CORN G compared to the corresponding LAM, while in the second study TPC was significantly different in yellow peppers only.

a)	TPC (mg GAE/g)	b)	TPC (mg GAE/g)
	Fresh samples		**Fresh samples**
LAM R	1,09 ± 0,04	**LAM R**	0,71 ± 0,05
CORN R	1,18 ± 0,04 *	**CORN R**	0,73 ± 0,10
LAM G	0,94 ± 0,07	**LAM G**	0,91 ± 0,06
CORN G	1,17 ± 0,13 *	**CORN G**	1,06 ± 0,06 **

Table 1. TPC of fresh peppers from the first (a) and second (b) harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (*p<0.05, ** p<0,01).*

After digestion, no differences in TPC were detected between CORN and LAM peppers in the TQ fractions, regardless the color and the harvesting period (Table 2).

a)	TPC (mg GAE/g)	b)	TPC (mg GAE/g)
	Digested TQ		**Digested TQ**
LAM R	0,51 ± 0,05	**LAM R**	0,45 ± 0,07
CORN R	0,53 ± 0,05	**CORN R**	0,37 ± 0,07
LAM G	0,66 ± 0,03	**LAM G**	0,53 ± 0,11
CORN G	0,64 ± 0,05	**CORN G**	0,56 ± 0,09

Table 2. TPC of TQ digested fraction of peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (ns).

Comparing digested <3K samples, in the first study a lower TPC was observed in CORN R than in LAM R. This difference was not confirmed in the second study. On the contrary, both studies evidenced a higher TPC in CORN G than LAM G (Table 3).

a)	TPC (mg GAE/g)	b)	TPC (mg GAE/g)
	Digested <3K		**Digested <3K**
LAM R	0,43 ± 0,04	**LAM R**	0,40 ± 0,05
CORN R	0,27 ± 0,01 ***	**CORN R**	0,35 ± 0,08
LAM G	0,49 ± 0,01	**LAM G**	0,39 ± 0,03
CORN G	0,61 ± 0,04 **	**CORN G**	0,45 ± 0,05 *

Table 3. TPC of <3k digested digested fraction of peppers from the first (a) and second (b) harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN with the corresponding LAM (*p<0,05;**p<0,01;***p<0.001).*

Digestibility and bioaccesibility indices based on TPC

Figure 2 shows digestibility and bioaccessibility indices based on TPC content. In peppers from the first harvesting period, the digestibility index appeared similar in the two cultivars of red peppers, while it was lowest in yellow CORN compared to LAM (Figure 2A). The bioaccessibility index was lower in CORN R than LAM R, while no differences were found between yellow peppers.

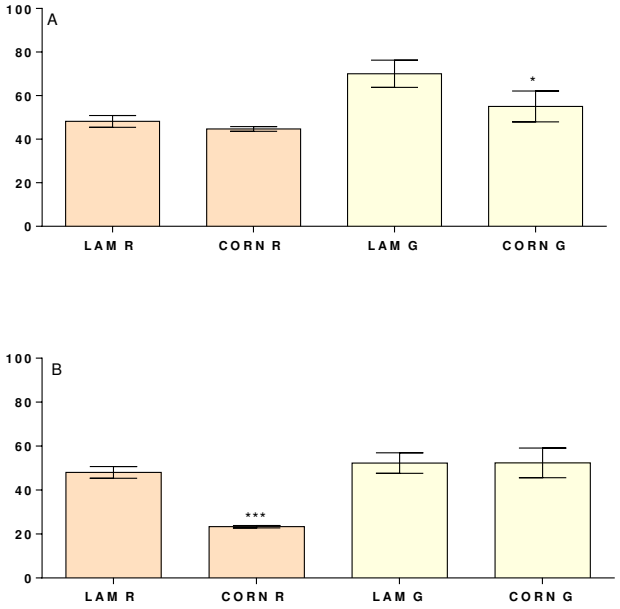

Figure 2. Digestibility (A) and Bioaccessibility(B) Indices based on TPC of peppers from the first harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN with the corresponding LAM (*p<0,05; ***p<0.001).*

Results obtained in peppers from the first harvesting period were confirmed only in part in peppers from the second harvesting period (Figure 3). In this case, both indices for red peppers were higher in LAM than CORN, while no differences were detected between yellow peppers.

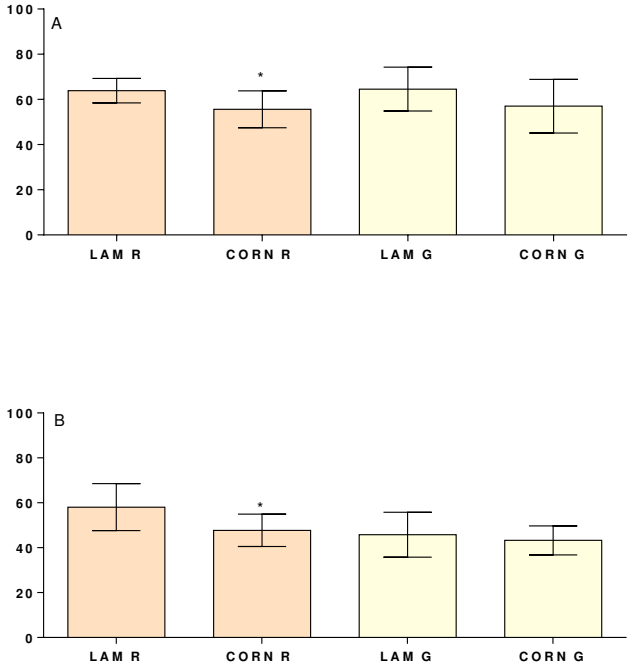

Figure 3. Digestibility (A) and Bioaccessibility(B) Indices based on TPC of peppers from the second harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (*p<0.05).*

Vitamin C content

In both studies, ascorbic acid concentration in fresh red and yellow peppers appeared similar comparing the two cultivars (Table 4).

a)	Vit. C (µmol Vit. C/g)	b)	Vit. C (µmol Vit. C/g)
	Fresh samples		**Fresh samples**
LAM R	6,30 ± 0,32	**LAM R**	4,89 ± 0,457
CORN R	6,45 ± 0,21	**CORN R**	4,95 ± 0,3
LAM G	5,18 ± 0,11	**LAM G**	6,66 ± 0,83
CORN G	5,33 ± 0,11	**CORN G**	7,56 ± 0,62

Table 4. Vitamin C content of fresh peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (ns)

In all samples, *in vitro* digestion resulted in the release of Vitamin C from the matrix. As shown in Table 5, in the first study Vitamin C concentration appeared significantly higher in TQ fractions of CORN R than LAM R, but this higher ascorbic acid release was not confirmed in the second study.

Comparing yellow peppers, in both studies a significantly higher Vitamin C content was detected in CORN than LAM.

a)	Vit. C (µmol Vit. C/g)	b)	Vit. C (µmol Vit. C/g)
	Digested TQ		**Digested TQ**
LAM R	6,68 ± 0,51	**LAM R**	3,68 ± 0,39
CORN R	8,80 ± 0,04 ***	**CORN R**	4,32 ± 0,70
LAM G	5,25 ± 0,38	**LAM G**	4,04 ± 0,71
CORN G	6,55 ± 0,71 *	**CORN G**	5,64 ± 0,97 *

Table 5. Vitamin C concentration in TQ digested fraction of peppers from the first (a) and second (b) harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (*p<0,05; ***p<0.001).*

Comparing the <3k digested fractions, no differences in Vitamin C content were detected (Table 6).

a)	Vit. C (µmol Vit. C/g)	b)	Vit. C (µmol Vit. C/g)
	Digested <3K		**Digested <3K**
LAM R	3,47 ± 0,33	**LAM R**	3,12 ± 0,7
CORN R	3,37 ± 0,07	**CORN R**	2,6 ± 0,57
LAM G	3,77 ± 0,07	**LAM G**	3,84 ± 0,73
CORN G	4,17 ± 0,39	**CORN G**	4,64 ± 0,46

Table 6. Vitamin C concentration in <3K digested fraction of peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (ns).

Digestibility and bioaccesibility indices based on Vitamin C content

In both studies, the digestibility index based on Vitamin C content was higher in CORN R than LAM R (Figure 4A and 5A, respectively). A highest digestibility index of CORN G compared with LAM G was observed in the first study (Figure 4A), but it was not confirmed in the second one (Figure 5A).

In the first study, a higher bioaccessibility index was evidenced in LAM R CORN R, and in CORN G than LAM G, while no differences were detected in the second study (Figure 4B and 5B).

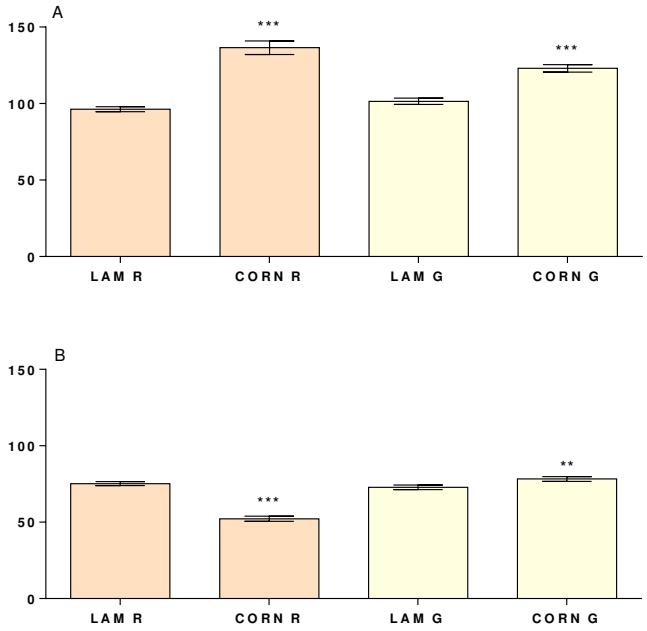

Figure 4. Digestibility (A) and Bioaccessibility(B) Indices based on Vitamin C concentration of peppers from the first harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (**p<0,01; ***p<0.001).*

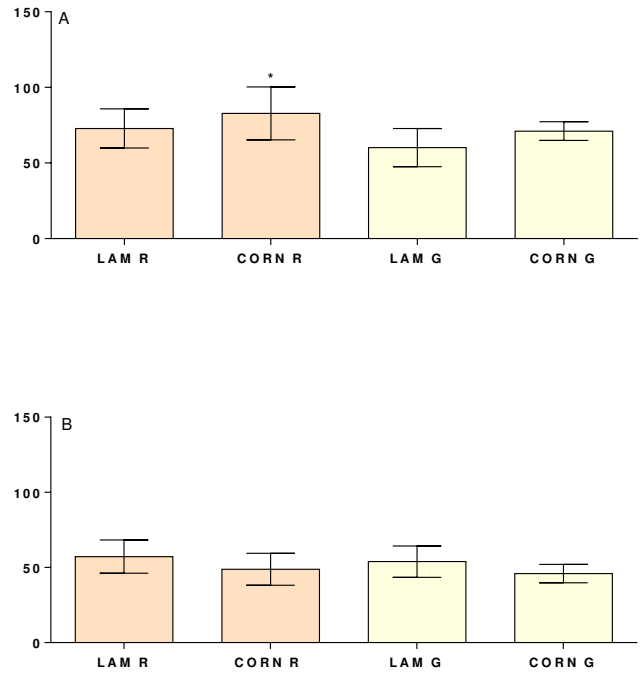

Figure 5. Digestibility (A) and Bioaccessibility(B) Indices based on vitamin C concentration of peppers from the second harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (*p<0.05).*

Total carotenoid content (TCC)

In fresh samples, no differences in TCC were detected between the two varieties of red peppers. In yellow peppers, TCC appeared higher in Lam than CORN from the first harvesting period, while it was the opposite in peppers from the second harvesting period (Table 7).

a)	**TCC (*µg*/g)**	**b)**	**TCC (*µg*/g)**
	Fresh samples		**Fresh samples**
LAM R	130,50 ± 55,27	**LAM R**	166,10 ± 17,59
CORN R	185,00 ± 25,33	**CORN R**	187,32 ± 10,74
LAM G	63,54 ± 8,62	**LAM G**	83,46 ± 12,57
CORN G	41,67 ± 3,59**	**CORN G**	117,92 ± 10,73 **

Table 7. TCC of fresh peppers from the first (a) and second (b) harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (** p<0,01).*

No differences were detected in the TQ digested fractions between the two varieties (Table 8).

In both studies, TCC in the <3K digested fraction was very low and below detection limit. This could be due to the low bioaccessibility of these molecules, as recently reported by Estevez-Santiago *et al* (23).

a)	**TCC (*µg*/g)**	**b)**	**TCC (*µg*/g)**
	Digested TQ		**Digested TQ**
LAM R	5,40 ± 1,69	**LAM R**	3,20 ± 0,14
CORN R	6,20 ± 0,08	**CORN R**	3,21 ± 0,28
LAM G	3,98 ± 0,76	**LAM G**	2,94 ± 0,32
CORN G	4,54 ± 0,98	**CORN G**	3,21 ± 0,19

Table 8. TCC of TQ digested fraction of peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN peppers with the corresponding LAM (ns).

Digestibility index based on TCC

In peppers from the first harvesting period, no differences in digestibility index based on TCC were found between cultivars (Figure 6). A highest digestibility index in yellow LAM compared to CORN was observed in the second study (Figure 7).

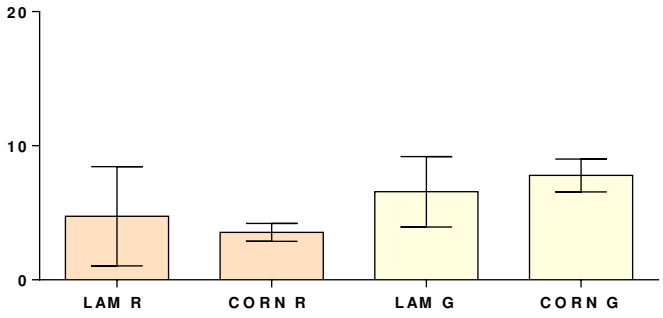

Figure 6. Digestibility Index based on TCC of peppers from the first harvesting period *Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (ns).*

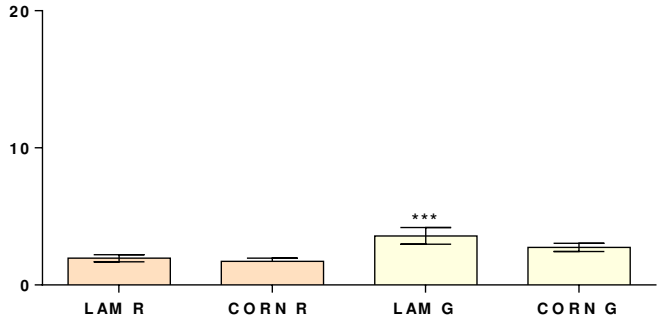

Figure 7. Digestibility Index based on TCC of peppers from the second harvesting period.
*Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (***p<0.001).*

Total antioxidant capacity (TAC)

In both studies, TAC of red peppers from the two varieties was similar. In the first study, TAC was similar also between the yellow peppers, while in the second study it was higher in CORN than LAM (Table 9).

a)	TAC (*µmol*TE/g)	b)	TAC (*µmol*TE/g)
	Fresh samples		**Fresh samples**
LAM R	8,02 ± 0,47	**LAM R**	4,002 ± 0,362
CORN R	8,63 ± 0,70	**CORN R**	4,530 ± 0,623
LAM G	6,85 ± 0,23	**LAM G**	5,029 ± 0,512
CORN G	7,2 ± 0,56	**CORN G**	6,163 ± 0,873 *

Table 9. TAC of fresh peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (p<0,05).*

As reported in Table 10 and 11, TAC hugely increased in TQ samples compared to the not digested counterparts. Differences highlighted among fresh products disappeared after digestion.

a)	TAC (*µmol*TE/g)	b)	TAC (*µmol*TE/g)
	Digested TQ		**Digested TQ**
LAM R	54,24 ± 6,61	**LAM R**	39,388 ± 3,539
CORN R	50,00 ± 4,65	**CORN R**	39,853 ± 3,137
LAM G	54,91 ± 0,83	**LAM G**	43,239 ± 1,953
CORN G	54,135 ± 2,6	**CORN G**	41,352 ± 2,127

Table 10. TAC of TQ digested fraction of peppers from the first (a) and second (b) harvesting period. *Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (ns).*

a)	TAC (*µmol*TE/g)	b)	TAC (*µmol*TE/g)
	Digested <3K		**Digested <3K**
LAM R	31,18 ± 3,3	**LAM R**	28,873 ± 055
CORN R	31,04 ± 1,89	**CORN R**	28,371 ± 0753
LAM G	34,25 ± 3,02	**LAM G**	27,616 ± 0,897
CORN G	32,44 ± 3,63	**CORN G**	26,249 ± 1,182

Table 11. TAC of <3k digested fraction of peppers from the first (a) and second (b) harvesting period.
Data are means ± SD. Statistical analysis was by the Student's t test to compare CORN red or yellow pepper with the corresponding LAM (ns).

Data of TAC in fresh and digested peppers were not used to calculate digestibility and bioaccessibility indices as results could be influenced by the presence of antioxidant compounds in the digestive juices and bile salts (24).

DISCUSSION

Health and well-being of consumers are major drivers of the modern food industry. There is an increased interest in the role that some molecules may play in preventing or ameliorating the effect of major diseases (for example, some types of cancer, cardiovascular diseases, eye disorders, among others). An important strategy could be the selection of cultivars with higher nutritional and healthy value, since it is known that the concentration of bioactive compounds may be different between cultivars of the same species (25). However, it is important to note that the proportion of an ingested compound that is made available for its intended mode of action is more relevant than the total amount present in the original food. Indeed, although the total amount of a healthy nutrient may be obtained from composition analysis, its availability for absorption in the gut is in many cases quite uncertain and can varies for the same food depending on several factors such as processing conditions, chemical state of the nutrient, presence of other components, disruption of the natural matrix or the microstructure created during processing (26). Therefore, when selecting cultivars, bioavailability of food components need to be taken into account.

Bioavailability, i.e. the proportion of a food constituent that is absorbed and utilized in the normal metabolism, depends on bioaccessibility, that is the amount of the food constituent that is released from the solid food matrix in the gastrointestinal tract (27).

The present work aimed to evaluate and to compare digestibility and bioavailability indices of the main functional component between Lamuyo and Corno di Toro peppers, also in relation to the harvesting periods.

Data clearly showed that phenolic compounds and TCC in fresh extracts were different in the various cultivars, in agreement with other studies in literature (28). The cultivar Corno di Toro showed higher TPC than LAM in both studies while carotenoid content was significant higher in CORN only in the second harvesting period. Overall red cultivar evidenced higher TCC then the yellow one. Several authors have identified capsanthin as the main carotenoid in different varieties of red peppers (5,29).

Among the different phenolic compounds, bioavailability appears to differ greatly and the most abundant ones in our diet do not necessarily correspond to those with best bioavailability profile.

Bioaccessible polyphenols were higher in Lamuyo peppers for the red type and in Cornelio for the yellow ones. The mechanical action with simulated mastication mediates the breakdown of fruits

cells with the release of these molecules contained in vacuoles and those linked weakly to the cell wall. In addition, acidic environment during the gastric phase contributes to the extraction of phenols from solid matrices (29-30). Digestibility and bioaccessibility indices were higher in Lamuyo peppers. This finding may be explained partially for a low molecular mass of their phenolic compounds, as previously reported and according to other authors (31-32).

TCC decreased a lot after digestion in all samples. Only a very low proportion of carotenoids has been reported to become bioaccesible (33). In some fruits (such as mango, papaya) carotenoids are found in oil droplets in chromoplast and hydroxycarotenoids are mostly esterified with fatty acids, being more easily extracted during digestion. Carotenoids bioavailability from foods varies greatly depending on endogenous (product-related) and exogenous (process-related) factors. Amount and type of fat present in the vicinity is a key factor that affects bioaccessibility. A minimum amount of fat is necessary for absorption, so formulation of carotenoids in an oily matrix may enhance higher bioaccessibility. Important steps in carotenoid absorption are release from the food matrix, micelle formation, uptake into mucosal cells, packing into chylomicrons, and transport within the lymphatic system (36). In this study Lamuyo fruit showed higher bioaccessibility index than CORN only in the second harvesting period.

Ascorbic acid concentration in fresh peppers appeared similar comparing the two cultivars but after digestion process Corno di Toro peppers (red and yellow) showed an higher digestibility and bioaccessibility indices than Lamuyo, suggesting a release of this nutrient from the food matrix.

Overall, the noticeable level of phenolic compounds, ascorbic acid as well as carotenoids contributes to antioxidant properties in pepper fruits (5,37). Some authors have suggested that the antioxidant activity from phenolic is related to differences in chemical structure as the number and positions of hydroxyl groups in the aromatic rings and the methoxy substituents in the ortho position to the OH (38-39). In this study, no differences in TAC where evidenced between the two cultivars. TAC hugely increased in TQ and <3k fractions compared to the not digested counterparts, depending not only on the release of bioactives during digestion but also on the presence of antioxidants in the digestive juices.

This work sets a new effective approach in the study of the nutritional properties of food. The use of *in vitro* digestion model allows to estimate the digestibility index, i.e. the efficiency of how a component is released from the food matrix by the action of the digestive enzymes. In addition, the bioaccessibility index indicates the percentage of a component, once solubilized, and released from the matrix, that has been brought to a size compatible with the intestinal absorption. This index represents an important nutritional value of food since it suggests the potential molecules that can exert biological activity in the body. To validate these data, it is necessary to repeat analysis by

considering a larger number of samples, because high variability is present in these cultivars. More studies are also needed to compare *in vivo* with *in vitro* results.

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FINAL CONSIDERATIONS

In the recent years, it has become evident that health benefits are associated with correct dietary choice. Fruits and vegetables are an essential part of the Mediterranean diet, and they contribute to the prevention of chronic and acute diseases.

Fruits and vegetables are a good sources of potentially bioactive molecules known as phytochemicals.

Many of the biological actions of phytochemicals have been generally related to their free radical scavenging and antioxidant capacity, but emerging findings seem to indicate that natural compounds may also act in increasing endogenous antioxidant defense. Epidemiological studies suggest that consumption of polyphenol-rich foods and beverages is associated with a reduced risk of cardiovascular diseases, diabetes and certain forms of cancer.

Different plant foods have great intrinsic variability in content and composition of these compounds, which concentration can also be affected by several factors as maturity stage or agronomic conditions. In addition, in processed food the technological treatment may have an impact on the overall content and bioaccessibility of phytochemicals.

At present, food is nutritionally evaluated on the basis of their chemical characteristics, without taking into account that their components must be released during digestion to exert functional effects, and that the food matrix can have a great impact on the entity of the release, and therefore on bioaccessibility.

Numerous mechanisms may be responsible for the bioaccessibility of food components, including physico-chemical factors such as pH, temperature and texture of the matrix and their solubility in gastrointestinal fluids. In addition, technological processing modifies concentration and bioaccessibility of the food component, mainly through changes in the cell wall structure and properties, thus it is important to identify suitable treatment able to preserve the nutritional value of products.

It is worth noting that foods are complex matrices in which components are not present alone, but with other molecules that could have additive, synergistic or antagonist effect. Therefore, the effect of the single, discrete bioactive could be different from the effect of the whole food in which it is embedded.

In this context, the present PhD thesis aimed to test the effectiveness of bioactive compounds in different plant foods to clarify several aspects of the complex relationship among bioactives and their synergism, food matrix, and processing. An *in vitro* model has been used to investigate the effect of digestion on the release of bioactives from the food matrix, allowing the determination of their bioaccessibility in a relatively inexpensive and technically reproducible way. The bioactivity

of bioaccessible molecules was then investigated, including also the use of a biological system for the evaluation of a possible protective role.

Three types of food were considered: mandarin juices prepared with different technological treatments, bread made with ancient and modern grains, and peppers of different varieties.

The first study clearly evidenced that differences in bioactivity observed in the digested mandarin juice and ascribable to the different processing, almost disappear when juices effectiveness is evaluated in a biological system. Mandarin juice supplementation can modify the cell response to an oxidative stress regardless the technological treatment used to obtain it.

On the contrary, the second study failed to discriminate bread made with ancient or modern grains based on their composition or bioactivity after digestion. In this case, significant differences were evidenced when cells were supplemented with the digested bread, confirming the potential health effects of ancient grains.

Results of the third study evidenced that the different digestibility and bioaccessibility of the main functional components in peppers were more related to the harvesting period than to the variety.

Besides specific results obtained in the different studies, data reported in this thesis underline the need to carefully assess the bioaccessibility of bioactive compounds, and its modification due to intrinsic and extrinsic factors. The experimental approach used in this study, combining in vitro digestion and the supplementation of the digesta to a biological system, can be very useful while studying the nutritional value of raw and processed food, and prior to *in vivo* investigation. Therefore, it could represent a useful tool to drive research and industry towards the improvement of the nutritional value of products.

APPENDIX

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COMPREHENSIVE REVIEW

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Ancient wheat and health: a legend or the reality? A review on KAMUT khorasan wheat

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ABSTRACT

After WWII, the industrialized agriculture selected modern varieties of *Triticum turgidum* spp. *durum* and spp. *aestivum* (durum wheat and common wheat) based on higher yields and technological characteristics. Nowadays, the use of whole ancient grains and pseudo cereals is considered nutritionally important. How ancient grains have positive effects is not entirely known, the fragmentation of the scientific knowledge being also related to the fact that ancient grains are not a homogeneous category. The KAMUT® trademark indicates a specific and ancient variety of grain (*Triticum turgidum* ssp. *turanicum*, commonly khorasan wheat), and guarantees certain attributes making studies sufficiently comparable. In this work, studies on KAMUT® khorasan wheat have been systematically reviewed, evidencing different aspects supporting its benefits. Although it is not possible to establish whether all ancient grains share these positive characteristics, in total or in part, this review provides further evidences supporting the consumption of ancient grains.

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KAMUT® khorasan wheat; ancient grains; whole grains; ancient wheat

Introduction

Grains are seeds from plants of the Gramineae family (such as wheat, corn, rice, barley, oat and rye) that have been the basis for human nutrition for thousands of years. Grains are fundamental for sustenance, both for their nutritional value and for their chemical properties that allow for a variety of uses in the food industry. Last, but not least, grains can be stored for long periods, and easily transported.

Wheat was one of the first domesticated food crops, and for about 8000 years it has been the basic staple food of the major civilizations of Europe, West Asia and North Africa. Today, wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans (Curtis 2002).

The most commonly used types of wheat, *Triticum turgidum* ssp. *durum* (or durum wheat), used to make pasta, and *Triticum turgidum* ssp. *aestivum* (or common wheat), used to make bread, originated thousands of years ago through naturally occurring hybridization of their progenitors. In the last 60 years, there has been an ever-increasing number of the varieties available, for both durum wheat and common wheat, while ancient varieties of this cereal have been largely forgotten or lost.

Ancient wheat is loosely defined as wheat that was used by ancient civilizations. Usually ancient wheat is considered to include einkorn, emmer, khorasan and spelt. Another term used to describe wheat commonly grown in the period between ancient wheat and modern wheat is heritage wheat. This wheat consists of varieties selected from either ancient wheat or wild wheat. Ancient wheat and heritage wheat generally consist of land races, which mean they were made up of many closely related strains. Land races have a huge diversity in their populations giving them great advantages in facing extremes in climate fluctuation and disease and insect pressure. The reason for this is that this diverse population contains strains which vary in their susceptibility to the aforementioned challenges. Modern wheat, by contrast is made up of homogeneous strains, which are the result of intensive breeding programs generally starting after WWII. During this period, the industrialization of agriculture began to include high inputs of

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chemicals to help increase yields and a focus on bread in and pasta production, which increased speed and efficacy of the process. Especially the size of the loaf of bread was of great importance therefore, one important goal was to increase the number of loaves of bread from each kg of flour used. So pure strains of wheat were developed with greater yield potential and greater loaf volume capacity.

In the last decade, some of these "ancient grains", not subjected to extensive genetic improvements, have been reintroduced, and the growing awareness regarding foods considered natural and healthy have further increased the interest in alternative cereals. This interest is also associated with the fact that some of them are reported to be better tolerated by individuals that suffer from intolerance or allergies to modern wheat (Molberg et al. 2005; Spaenij-Dekking et al. 2005).

Ancient grains (khorasan wheat, barley, spelt, rye, millet, oat and sorghum) and pseudo cereals (i.e. quinoa, amaranth and buckwheat) are considered healthy due to their higher content of certain components (Wijngaard & Arendt 2006) and to their common use as whole grains. Whole grains contain higher amounts of positive components compared to refined grains. Most importantly, dietary fiber, vitamins and minerals, but also other bioactive molecules such as omega 3 fatty acids, prebiotic oligosaccharides, phytosterols, polyphenols, etc., and probably the interaction of all the components rather than each individual one gives whole grains their nutritional value (Slavin et al. 2001).

Epidemiological studies have scientifically proven that regular eating of whole grains positively affects human health, because it reduces the risk of type 2 diabetes (Maki & Phillips 2015) and manages obesity (Giacco et al. 2011). It is also linked to both a lower cardiovascular mortality rate in the elderly and a reduction in colon cancer cases (Truswell 2002; Sahyoun et al. 2006; Gil et al. 2011).

Despite dietary guidelines all over the world are recommending the inclusion of whole grains, the knowledge of the healthy effect of whole ancient grains is fragmented and based more on the evaluation of the properties of the main chemical components than on the effect of the individual ancient grain on those who have ingested it. In addition, compositional differences existing among different ancient grains and among varieties of the same grain (Gawlik-Dziki et al. 2012; Carvalho et al. 2015), and the strong influence of agronomic and environmental factors on the level of phytochemicals in plants (Danesi et al. 2014) could make difficult to generalize results obtained in a specific study.

In this respect, KAMUT® khorasan wheat represents an interesting exception, since it is a specific and ancient variety of grain (*Triticum turgidum* ssp. *turanicum*, commonly called khorasan wheat). KAMUT® is a registered trademark of Kamut International, Ltd. (Big Sandy, MT) and Kamut Enterprises of Europe (Oudenaarde, Belgium), bvba, and the trademark guarantees certain attributes, mainly a protein content of 12–18% and a selenium content between 400 and 1000 ppb, and several quality specifications related to growing conditions. For example, the grain must be always grown certified organic and never hybridized or genetically modified (Quinn 1999). This makes possible the comparison among studies. The chemical composition of KAMUT® khorasan wheat, durum wheat and common wheat is reported in Table 1.

Table 1. Chemical composition and energy of KAMUT® khorasan wheat, common wheat and durum wheat.

	KAMUT® khorasan wheat	Soft wheat	Durum wheat
Water (g/100 g)	11.07	10.42	10.94
Energy (Kcal/100 g)	337	340	339
Proteins (g/100 g)	14.54	10.69	13.68
Total lipid fat (g/100 g)	2.13	1.99	2.47
Saturated (g/100 g)	0.196	0.368	0.454
Monounsaturated (g/100 g)	0.213	0.227	0.344
Polyunsaturated (g/100 g)	0.621	0.837	0.978
Cholesterol (mg/100 g)	0	0	0
Carbohydrate (g/100 g)	70.58	75.36	71.13
Fibres total (g/100 g)	11.1	12.7	n.d.
Sugars (g/100 g)	7.84	0.41	n.d.
Vitamin C (mg/100 g)	0	0	0
Thiamine (mg/100 g)	0.566	0.410	0.419
Riboflavin (mg/100 g)	0.184	0.107	0.121
Niacin (mg/100 g)	6.375	4.766	6.738
Vitamin B_6 (mg/100 g)	0.259	0.378	0.419
Folic acid (µg/100 g)	n.d.	41	43
Vitamin B_{12} (µg/100 g)	n.d.	0	0
Vitamin A (µg/100 g)	1	0	0
Vitamin E (mg/100 g)	0.61	1.01	n.d.
Vitamin D (µg/100 g)	n.d.	0	0
Vitamin K (µg/100 g)	1.8	1.9	n.d.
Calcium (mg/100 g)	22	34	34
Iron (mg/100 g)	3.77	5.37	3.52
Magnesium (mg/100 g)	130	90	144
Phosphorus (mg/100 g)	364	402	508
Potassium (mg/100 g)	403	435	431
Sodium (mg/100 g)	5	2	2
Zinc (mg/100 g)	3.68	3.46	4.16

United States Department of Agriculture. USDA Food Composition Database. Available from: http://ndb.nal.usda.gov/.

In this work, studies performed to evaluate the nutritional, technological and healthy characteristics of KAMUT® khorasan wheat compared to modern wheat have been systematically reviewed, in the attempt to go deeper inside the scientific basis for the possible exploitation of this ancient grain to produce food having an enhanced nutritional value.

Search strategy

The detailed selection process is presented in Figure 1. First access in PubMed was performed on 30

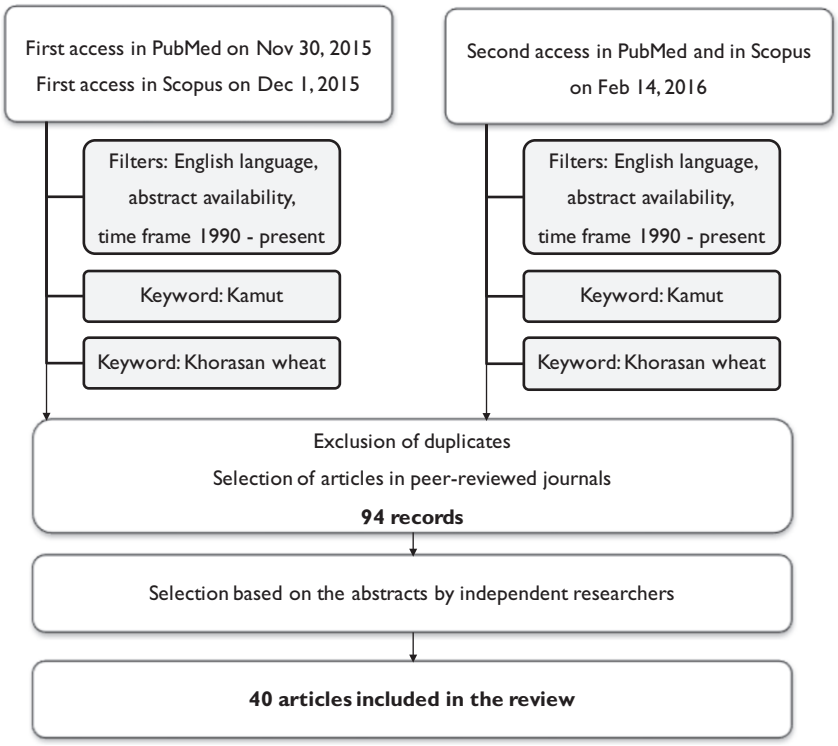

Figure 1. Flow diagram of search strategy and study selection.

November 2015 using "Kamut" as keyword and English language, abstract availability and publication in the year 1990-present as filters. The timeframe period of the search was selected based on the year of registration of KAMUT® as a trademark. Twenty-two records were retrieved. Search was performed again using "Khorasan wheat" as keyword and the same filters, and it retrieved 12 records, six of them in common with the previous search. PubMed search was performed again on 10 February 2016, and four additional records, three for Kamut and one for khorasan wheat were added to the list. First search on Scopus was performed on 1 December 2015 using the same keywords and filters. A second search was performed on 10 February 2016.

Lists were compared to avoid duplicates, and articles published in peer-reviewed journals were selected, so obtaining 94 records. Records were then checked based on their abstract by independent researchers, and those out of the scope of this review, as well as articles reporting data on khorasan wheat but not specifically on KAMUT® wheat were excluded. In the end, 40 articles were included in the review.

Results and discussion

Technological and nutritional aspects

The organoleptic and nutritional properties of grain products depend on the flour used for their production. The physical result of the flour is extremely important in the final product, especially in baked goods, and therefore one of the primary limitations in the use of flour made from something other than wheat is its inadequate chemical properties. The partial or complete substitution of normal flour with flour from ancient grains could add nutritional value to the final products, provided that the physical and sensory characteristics of the substituting flour are equal or better than those of wheat so that public acceptance is not deterred.

The suitability of KAMUT® khorasan wheat has been positively ascertained in the production of bread (Piergiovanni et al. 2009), tortillas (Carini et al. 2010) and cookies (Chandi et al. 2015). In tortillas, the substitution of regular flour with KAMUT® khorasan wheat flour slightly modified flour reaction to water (Serventi et al. 2009), but the physiochemical properties of the finished product were the same, even in products with a long shelf-life (180 days) (Carini et al. 2010). In cookie production, the flour made from KAMUT® khorasan wheat appeared to be able to substitute common wheat for up to 50% without causing qualitative physical alterations in the product's properties (Chandi et al. 2015). Furthermore, bread made with a mix of ancient cereals, including KAMUT® khorasan wheat, demonstrated comparable sensorial and physical properties as that of wheat flour

(Angioloni & Collar 2011). The physicochemical and metabolomic characteristics of KAMUT® khorasan and durum wheat fermented dough were investigated by Balestra et al. (2015), who found KAMUT® flour to be more suitable than durum wheat for the fermentation processes tested, especially at acidic conditions.

Studies reported above indicate that KAMUT® khorasan wheat enjoys great versatility as a raw material because it is suitable for several consumer uses. In addition, consumers enjoy products made with the KAMUT® khorasan wheat (Holmer et al. 2012), and appreciate numerous quality attributes (e.g. it is organically grown, it is managed according to a global value-enhancement strategy) featured by KAMUT® wheat (Canavari et al. 2009).

According to Canavari et al. (2009), Italian large-scale retail chains are deeply interested in marketing this type of product. At present, Italy is the largest EU consumer of KAMUT® and imports approximately 70% of all the KAMUT® wheat exported in Europe. Most of the Italian KAMUT®-based products are exported into other EU countries, while in Germany, France and Belgium nearly all the imported KAMUT® grains are domestically consumed.

The use of KAMUT® khorasan wheat flour as a substitute for other ingredients can contribute to the improvement of the nutritional value of the final product. Bread made with KAMUT® khorasan wheat flour had more carotenoid and was richer in protein compared to breads made with modern wheat (Pasqualone et al. 2011). Similarly, total phenolics, total flavonoids and antioxidant capacity were higher in spelt and KAMUT® flakes and muesli than in corresponding conventional products, although lower than in products made with Dickopf wheat and red wheat (Sumczynski et al. 2015). In addition, products made with spelt and KAMUT® wheat had the highest protein level (Sumczynski et al. 2015).

Shewry and Hey (2015) carried out an extensive literature review in order to determine whether ancient wheat species differ from common wheat in a range of components that have established or proposed benefits to human health. Among studies included in the review, Abdel-Aal el and Rabalski (2008) reported a higher concentration of total phenolics in KAMUT® wheat than in 10 common wheat cultivars. This could be due to the low polyphenol oxidase found in KAMUT® flour compared to other 59 whole meal flours (Hidalgo et al. 2013). In addition, a higher content of total carotenoids in KAMUT® wheat compared with common wheat was reported (Abdel-Aal el et al. 2007). The major component was lutein, which was present at 5.77 mg/g concentration compared with a mean of 2.06 mg/g in four common wheat cultivars. The high content of lutein was confirmed by other studies (Abdel-Aal el et al. 2002; Hidalgo et al. 2006; Abdel-Aal el & Rabalski 2008). On the contrary, total tocols were lower in KAMUT® wheat than common wheat cultivars (Hidalgo et al. 2006; Abdel-Aal el & Rabalski 2008).

The evaluation of the functional components of 10 Italian durum wheat cultivars highlighted remarkable differences between modern and old genotypes (Dinelli et al. 2009). Besides no significant differences among investigated cultivars were detected as regards the amounts of total phenolic and flavonoid compounds, the qualitative phytochemical profile between old and modern varieties was remarkably diverse. Ancient wheat varieties showed a mean number of phenolic compounds and isomer forms significantly higher than in modern genotypes. As examples, coumarin was detected only in the free phenolic fraction of the old wheat genotype KAMUT® khorasan, and procyanidin B3 and occurred in the free phenolics of Iride and KAMUT® khorasan wheat.

The putative functionality of KAMUT® khorasan wheat could be not only connected to its high content of phenols and carotenoids, but also to the presence of other molecules such as bioactive peptides, small protein fragments that have positive effects on body functions in humans (Kitts & Weiler 2003). In the study by Coda et al. (2012), a pool of selected lactic acid bacteria was used for the sourdough fermentation of various cereal flours. The highest radical-scavenging activity of water/salt-soluble extracts was found for whole wheat, spelt, rye and KAMUT® sourdoughs demonstrating that selected lactic acid bacteria have the capacity to synthesize antioxidant peptides during the sourdough fermentation of these cereal flours.

The health-promoting effects of wholemeal flours could be related to the presence of other minor components. Pedersen et al. (2011) evidenced the presence of benzoxazinoids, a group of natural compounds having documented physiological effects, in hydrothermally processed grains of KAMUT®, a commercial variety of rye (*Secale cereale* cv. Picasso) and an old Nordic rye landrace (*Secale cereale*, Svedjerug), as well as in bread baked with flour milled from those grains.

There is ample evidence that diet can modulate both composition and functionality of the human gut microbiota, in a complex and dynamic interplay crucial for maintaining the host-microbiota mutualism (Cotillard et al. 2013). KAMUT® wheat could be a special raw material for improving the prebiotic properties of wheat-based products. Although the content of soluble dietary fiber was found lower in KAMUT®

flour than in grains of *Triticum polonicum* (average of nine spring lines) (Wiwart et al. 2013), it was higher than in the Italian modern durum wheat variety Claudio (Di Silvestro et al. 2014). In addition, KAMUT® fibers have been shown to have a prebiotic effect and to promote the growth of Lactobacillus and Bifidobacterium (Marotti et al. 2012). Taneyo Saa et al. (2014) described for the first time the effect of KAMUT® khorasan wheat on the human gut microbial ecology. According to their results, the KAMUT® khorasan-based diet was mainly characterized by the release of short fatty acids and phenol compounds, as well as by a slight increase in health-promoting mutualists of the gut microbiota in comparison to whole durum wheat adopted as a control diet.

In vitro and animal studies

In the study by Valli et al. (2016), cookies baked with three different whole grains flours (KAMUT® khorasan wheat grown in North America, khorasan wheat grown in Italy, and a modern durum wheat) and two fermentation methods (standard and lactic fermentation) were digested *in vitro* and supplemented to cultured liver cells. Cells were then exposed to either an oxidative or an inflammatory stress by adding H_2O_2 or lipopolysaccharides. Overall, cell supplementation with the bioaccessible fraction of all digested cookies 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).

The aim of the study by Gianotti et al. (2011) was to evaluate in rats whether a diet comprised exclusively of bread made from whole modern durum flour or KAMUT® khorasan wheat flour could affect the response to the oxidative stress induced by the administration of doxorubicin. Two different bread-making processes were used for whole grain KAMUT® khorasan, sourdough and baker's yeast, while whole grain durum wheat bread was made using standard fermentation (baker's yeast) only. The authors concluded that diet based on the ancient cereal is able to supply a variety of nutrients and bioactive components that improve the organism's ability to defend itself against oxidative stress, independent of the type of fermentation used to make the bread. Using a similar experimental design, Benedetti et al. (2012) confirmed these findings and demonstrated that a diet based on bread made from KAMUT® khorasan wheat is able to increase plasma antioxidant concentration and antioxidant enzyme activity.

In addition, histologic tests on the liver evidenced an inflammatory status in rats fed modern durum wheat and not in rats fed KAMUT® khorasan wheat. Feeding rats with pasta made from KAMUT® khorasan wheat or durum wheat obtained similar results (Carnevali et al. 2014). After 7 weeks, all of the rats fed modern durum wheat pasta showed alteration in the morphology of their duodenums' mucosa, with an unusual flattening of the intestinal villus and infiltration of lymphocytes, and an increased volume of lymph follicles in the spleen and lymph nodes. These signs of inflammation were not present in the rats fed pasta made from KAMUT® khorasan wheat.

Human intervention trials

Five intervention trials involving human volunteers are reported in the literature. The first one (Scazzina et al. 2008) evidenced that the incorporation of carrots, soy, and whole KAMUT® meal in a standard wheat tortillas formulation results in a product with a lower glycaemic index (GI) and a relatively high total antioxidant capacity. However, the GI of tortillas made with KAMUT® only did not differ from standard tortillas, suggesting the main contribution or the synergistic action of other ingredients.

In the other trials, products made from KAMUT® khorasan wheat were compared to products made with modern common and durum wheat. Both the KAMUT® khorasan and the control wheat were cultivated in organic agriculture. Semi-whole wheat semolina and flour from KAMUT® and modern wheat were similarly processed to obtain pasta and baked products. All studies were randomized, double-blinded, crossover trials with two intervention phases in which subjects were assigned to consume either the KAMUT® or the control wheat.

The first study (Sofi et al. 2013) involved 22 healthy volunteers carrying risk factors for cardiovascular diseases. Volunteers were randomly divided into two groups, assigned to consume the KAMUT® khorasan or control grain products made from organic semi-whole-wheat for 8 weeks. Then, after an 8-weeks washout, groups were crossed over for additional 8 weeks. The consumption of products made with KAMUT® khorasan wheat resulted in a significant reduction in blood total cholesterol (–4.0%), LDL cholesterol (–7.8%) and glucose levels (from 81.1 to 78.1 mg/dL). Redox status, measured by the blood level of thiobarbituric acid reactive substances (TBARS) and carbonyl levels was significantly improved only after the KAMUT® intervention phase. Furthermore, consumption of KAMUT® khorasan

products resulted in a significant decrease of the level of pro-inflammatory cytokines: tumor necrosis factor α (TNFα, –34.6%), interleukin 6 (IL6, –23.6%), interleukin 12 (IL12, –28.1%) and vascular endothelial growth factor (VEGF, −10.5%). No changes were observed for the same patients after eating the control products made from modern wheat.

In the second study (Sofi et al. 2014), 20 participants classified with moderate inflammatory bowel syndrome (IBS) were divided into two groups, the first receiving KAMUT® khorasan products and the second modern wheat products for 6 weeks. After a 6-week washout period, volunteers were crossed over for additional 6 weeks. The IBS-GAI (Global Assessment of Improvement) and the IBS-SSS (Symptom Severity Scale) were used to evaluate IBS symptoms, and evidenced significant improvements in patients consuming KAMUT® khorasan products. A concomitant significant reduction in circulatory pro-inflammatory cytokine levels, including interleukin 6 (–36.2%), interleukin 17 (–23.3%), interferon γ (–33.6%) and VEGF (–23.7%) was detected after the KAMUT® khorasan wheat intervention phase.

The third trial (Whittaker et al. 2015) involved 22 patients diagnosed with acute coronary syndrome with a cross over study design with two intervention phases (8 weeks each, with an 8 week wash-out period) in which subjects were assigned to consume either the KAMUT® khorasan or the control wheat. Even in this study consumption of products made with KAMUT® khorasan wheat resulted in a significant amelioration of blood total cholesterol (–6.8%), LDL cholesterol (–8.1%), glucose (–8.0%) and insulin level (–24.6%) from baseline levels. Moreover, a significant reduction in reactive oxygen species (ROS), lipoperoxidation of circulating monocytes and lymphocytes, and circulating TNFα was detected after consumption of KAMUT® products, while no changes were observed after consumption of modern wheat products.

Last, the study by Whittaker et al. (2016) was a randomized, double-blinded, crossover trial aimed at testing whether a replacement diet with KAMUT® khorasan wheat products and/or control wheat products could provide additive benefits to type 2 diabetes mellitus patients. Even in this study, compared to baseline a reduction in blood total (–3.7%) and LDL cholesterol (–3.4%), insulin (–16.3%) and glucose (–9.1%), as well as a significant reduction in circulating levels of ROS, VEGF, and interleukin 1 receptor antagonist (IL1Ra) were observed after consumption of KAMUT® products. No significant differences from baseline were noted after the modern wheat intervention phase.

Celiac disease and non-coeliac gluten sensitivity

Celiac disease (CD) is a chronic autoimmune disease of the intestine caused by exposure to gluten in genetically predisposed subjects (Ludvigsson et al. 2013). In Europe, South America, Australasia and the USA, between 0.5% and 1% of the population are affected, and a high percentage of celiac cases goes undiagnosed because of the large variety of symptoms (Martucci et al. 2002). The only treatment for CD is eliminating gluten from the diet. However, this is very difficult because in many food products other than pasta and baked goods contain gluten, which is also used as an excipient in drugs and vitamin supplements (van den Broeck et al. 2010). Furthermore, gluten-free products (GFPs) are considered of lower quality and poorer nutritional value compared to the gluten-containing counterparts. GFPs often have a greater carbohydrate and lipid content than their gluten containing equivalents, and some commercially available GFPs have a lower content of folates, iron and B vitamins. In addition, some studies have reported that GFD is associated with a lower intake of dietary fibre (Penagini et al. 2013).

At present, it is unknown if all wheat varieties are equally toxic to individuals with CD. In an attempt to identify grains less toxic to celiac patients, several scientists strongly focused on the analysis of grains considered forerunners of modern grains. Gregorini et al. (2009) and Colomba and Gregorini (2012) reported that both Graziella Ra and KAMUT® khorasan wheat are CD toxic as the modern durum accessions, and contain greater amounts of α-gliadin. Similarly, results by Šuligoj et al. (2013) underlined strongly the need for all cereals from the tribe Triticeae to be considered CD toxic.

Notwithstanding, KAMUT® khorasan wheat has been showed to have a lower percentage of epitopes than Senatore Cappelli, a heritage durum wheat selected and introduced over 100 years ago, and modern Claudio durum wheat and Manitoba common wheat (Valerii et al. 2015). The concentration of gliadin proteins carrying allergenic epitopes among the total protein pattern can influence the inflammatory response. Valerii et al. (2015) evidenced that wheat proteins induce an overactivation of the pro-inflammatory chemokine (C-X-C motif chemokine 10, CXCL10) in cultured peripheral blood mononucleated cells (PBMC) from subjects with non-celiac gluten sensitivity (NCGS), and overactivation level depends on the cereal source from which proteins are obtained. In this study, chemokine CXCL10 activation was higher after exposure to modern than ancient grain

protein. This could explain, at least in part, why KAMUT® wheat is reported to be better tolerated by individuals suffering from NCGS (Molberg et al. 2005; Spaenij-Dekking et al. 2005).

NCGS is characterized by intestinal and extra intestinal symptoms that occur after the ingestion of gluten-containing food in subjects in whom CD and wheat allergy have been ruled out (Tovoli et al. 2015). Gluten may not be the only triggers of NCGS, and different wheat proteins such as wheat amylase and trypsin inhibitors could contribute to the origination of symptoms (Inomata 2009).

Fermentable oligosaccharides, disaccharides, and monosaccharides and polyols (FODMAPs) can provoke gastrointestinal symptoms through mechanisms involving gut microbiota, gas production and fermentation (Halmos et al. 2014). Some grains and cereals are particularly rich in FODMAPs, and recent studies have shown that a diet low in FODMAPs results in improved symptoms in NCGS patients, supporting the hypothesis of a major role of FODMAPs compared to gluten (Biesiekierski et al. 2013). Although whole-grain flour from ancient wheat inhibited yeast fermentation, fructan levels were reported similar bread and pasta made with KAMUT® khorasan wheat and emmer and with modern wheat (common wheat; durum) (Gélinas et al. 2016).

Conclusions

The development of studies and research aimed to test the effectiveness of preventive and protective nutrients and food components has clarified many aspects of the complex relationship between nutrition and well-being. Notwithstanding, often a few things are forgotten, first that our diet is based on foods and not on individual molecules. If on one hand it is useful to prove that a certain component has a positive effect in the prevention of a disease, it is also important to identify which foods contain it. Foods are complex matrices in which that component is not present alone, but along with many other molecules that could have additive, synergistic or antagonist effect. In addition, processing often modifies concentration and bioavailability of the component. Finally, yet importantly, foods having a high consumption frequency have the highest possibility to allow the introduction of the effective dose of the component. In one word, the relationship among food components, food and health must be studied with a foodomics vision (Bordoni & Capozzi 2014).

Whole ancient grains in general, and KAMUT® khorasan wheat in particular, are an example of synergism among different components (Gianotti et al. 2011), and can be transformed into a large variety of products that are consumed every day. Studies reported in this review point out the health-promoting properties of KAMUT® khorasan wheat, not evident in commercial modern varieties. At this stage, it is not possible to establish whether the health effects are specific for KAMUT® khorasan wheat or all ancient grains share them. At present, further scientific evidences are needed to consider KAMUT® khorasan-based products as functional foods, but results are promising and there are several elements of great interest that challenge the scientific community to deepen the scientific knowledge about this ancient grain in particular and ancient grains in general.

Disclosure statement

The authors report no potential conflict of interest.

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